From Pores to Rafts to Toll-The Adventures of *L. monocytogenes* on Host Cell Membranes

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1 Introduction

The Gram-positive bacteria *Listeria monocytogenes* is the food borne etiological agent of Listeriosis whose manifestations include septicemia, meningitis (or meningoencephalitis), encephalitis and abortions (1). Although Listeriosis targets mainly immunocompromised populations such as pregnant women and fetus, the elderly, diabetics, AIDS or cancer patients, healthy individuals can also develop the disease, particularly if the foodstuff is heavily contaminated with highly virulent variants of the pathogen (2). Although almost all *Listeria* strains that induce sepsis, meningitis and encephalitis, as well as many other manifestations particularly in immunocompromised individuals are susceptible to most of the common antibiotics, the cure rate is only approximately 70% (2). Thus, if unrecognized and not treated in time, Listeriosis is associated with rates of fatality as high as 70 %. In addition, Listeriosis causes serious problems in livestock farming with 10% of animal death and 50% abortion being due to *Listeria* infection (3). Thus understanding the pathogenic mechanisms of *L. monocytogenes* is of high biomedical importance. Since it is well characterized and one of the most easily manipulated bacterial pathogens, *L. monocytogenes* is also a ´favourite` model pathogen for immunologists and microbiologists commonly used for characterizing the mammalian immune system and the pathogenic mechanisms of intracellular microorganisms.

1.1 Target host cells of *L. monocytogenes*

The natural route of infection with *L. monocytogenes* is through the gastrointestinal tract. Upon ingestion, bacteria invade the intestinal epithelium and /or Peyer´s patches, and disseminate via the bloodstream to the liver and spleen (1). Multiple components of the immune system are involved in protection from *Listeria* infection. Innate immunity controls pathogen replication during the first 2-3 days after infection. Early host defenses either eliminate the microbe or modify the slope of the growth curve, thereby preventing overwhelming sepsis and setting the stage for T-cell dependent elimination of the pathogen (4). The significance of innate immunity to *L. monocytogenes* is emphasized by the remarkable ability of mice deficient in both T-cell and humoral immunity to control the early phase of infection (5;6). The main target host cells involved in the survival as well as control of *L. monocytogenes* are discussed below.

1.1.1 Macrophages and Dendritic cells (DCs)

1.1.1.1 Kupffer cells

Upon intravenous inoculation, more than 60% of the *Listeria* inoculum is cleared from the bloodstream by the liver within 10 min (7). This is due to the Kupffer cells. Kupffer cells are resident tissue macrophages of the liver and account for 80-90% of the total fixed tissue macrophages in the body (4). Kupffer cells are more dense in the periportal region, and are optimally located for response to systemic bacteria and bacterial products transported from the gut to the liver via the portal vein (8;9). Mice deficient in Kupffer cells exhibit significant increases in blood *Listeria* and decreases in liver *Listeria* 10 min after injection (4). Thus, one of the very early actions of Kupffer cells is to physically trap the majority of *Listeria* in the liver during systemic infections. Since more that 90% of liver bateria in the later stage of infection are associated not with Kupffer cells but hepatocytes, (7;10), it has been suggested that Kupffer cells clear bacteria via adherence rather than phogocytosis (4). In addition, Kuppfer cells can also inhibit *Listeria* growth in by stander cells *in vivo,* either by physical interaction or secretion of soluble factors, including interleukin (IL)-6, IL-12 IL-1α, tumor necrosis factor alpha (TNF-α) and nitric oxide (NO). These mediators are capable of promoting proinflammatory responses and antimicrobial activity of other cell populations within the liver (4;11;12).

1.1.1.2 Resident splenic macrophages

Rapid removal of pathogens from the circulation by secondary lymphoid organs is requisite for successful control of infection. Blood-borne antigens are trapped mainly in the splenic marginal zone. Selective depletion of marginal zone macrophages and marginal metallophilic macrophages caused impaired control of infection. Depletion these cells did not however, limit Ag presentation since *Listeria*-specific unimpaired protective T cell immunity was induced (13). Therefore, marginal zone macrophages and marginal metallophilic macrophages are crucial for trapping of particulate Ag but dispensable for Ag presentation (13). Muraille et al (14) have shown that $CD8^+$ T cell priming is indeed mediated by dendritic cells rather than macrophages. However, since unpublished data by Jablonska et al show that *Listeria* infects and induces the expression of the CC-chemokine ligand (CCL2) in a $ERT9^+$ splenic macrophage

subtype it seems possible that listerial clearence by splenic macrophages is mediated via direct as well as indirect mechanisms.

1.1.1.3 Infiltrating monocytes

The recruitment of monocytes is a feature of the inflammatory response to infection with *Listeria* and is essential for bacteria clearance. Circulating monocytes express CCR2, the chemokine receptor for CCL2 which as mentioned above is induced in macrophages by *L. monocytogenes*. Mice that lack CCR2 or CCL2 show a markedly increased susceptibility to infection to *L. monocytogenes*. Such mice have considerably lower levels of TNF-α and inducible nitric oxide oxygen synthase (iNOS), factors that are essential for defence against *L. monocytogenes* infection (15). The role of macrophages in bacterial clearance was also demonstrated by the fact that when their recruitment to the sites of infection was blocked using a monoclonal antibody against the complement receptor 3 (also known as CD11b-CD18), the mice became more susceptible to *Listeria* infection (16).

1.1.1.4 Dendritic cells (DCs)

Although DCs play a major role in antigen presentation and generation of antilisterial T-cell responses, several studies show that DC are not a significant *in vivo* reservoir of *L. monocytogenes* (14;17). It is therefore proposed that the acquisition and presentation of *Listeria* antigen occurs via cross-presentation, a process that involves engulfment and degradation of infected cells (18). In addition to priming T cell responses, which are required for sterile clearance of *Listeria,* some subsets of DCs are also involved in the innate immune responses against *Listeria*. As mentioned above, TNF-α and iNOS are essential for defense against *Listeria* infection. Analysis of spleens from infected wild-type mice revealed a DC population that produces high levels of TNF-α and iNOS and these cells are absent from CCR2 deficient mice. These observation indicate that the newly discovered population of TNF-α and iNOS-producing DCs (known as TipDCs) is involved in the control of *Listeria* growth *in vivo* (19). It is worth to mentioning that $CD8\alpha^+$ lymphoid DCs have also been implicated as an important innate source of IFN-β during *Listeria* infection (20).

1.1.2 Granulocytes

1.1.2.1 Neutrophils

In mice, neutrophils represent approximately 15% of peripheral white blood cells. During bacterial infection, neutrophils efflux from the bone marrow into the peripheral blood, then into the sites of infection. Once localized at the site of infection, neutrophils may contribute to antibacterial resistance by killing bacteria, lysing infected host cells, stimulating apoptosis in infected cells, and / or secreting cytokines that suppress replication of intracellular bacteria within infected cells.

That neutrophils play a part in resistance to *Listeria* infections is demonstrated by the fact that neutrophil-deficient mice, as well as mice unable to mobilize neutrophils upon infection, exhibit dramatic increases of Listeria in the liver and spleen (see results section and (21;22).

1.1.2.2 Mast cells

Mast cells are derivatives of hematopoietic progenitor cells that migrate into virtually all vascularized tissues, where they complete their maturation (23). Mature mast cells normally reside close to epithelia, blood vessels, nerves, near smooth muscle cells, mucus-producing glands, in the airways and in the gastrointestinal tract (23). Under certain circumstances, morphologically identifiable mast cells can migrate locally within tissues, including into epithelia (24;25). In some species, including murine rodents, mast cells also occur within mesothelium-lined cavities, such as the peritoneal cavity (23;24).

Mast cells are known mainly for their involvement in mediating various harmful inflammatory reactions in the host, the best known of these being immunoglobulin E (IgE)-mediated immediate-type hypersensitivity reactions. Other pathologic processes involving mast cells include inflammatory bowel disease, autoimmune diseases, tissue remodeling and fibrosis (26). The contribution of mast cells to these chronic inflammatory conditions is exacerbated by the fact that mast cells are extremely long lived, with a lifespan of months to years, and because of their capacity to proliferate at sites of inflammation (27).

Although the focus on mast cells has largely been in relation to allergic or autoimmune diseases, their role in mediating bacterial clearance at sites of infection is now well recognized (28). After activation, mast cells exert their biological effects

by releasing preformed and *de novo* synthesized mediators such as histamine, proteases, leukotrienes, prostaglandins and various cytokines, including tumor necrosis factor (TNF-α). Although blood monocytes, tissue macrophages and Kupffer cells of liver are the best known sources of TNF-α, mast cells are the only cell type capable of storing presynthesized TNF-α (29;30). Because of this unique capability, mast cells provide the only readily available source of TNF-α within peripheral tissues during the early course of infection. Indeed, mast cells secrete TNF-α within minutes of bacterial challenge (31) while the secretion of TNF-α by other cell types is greatly delayed by the time required to complete *de novo* synthesis of this potent cytokine.
Additionally, the capacity of mast cells to ingest and kill opsonized bacteria led to the proposal that mast cells have a physiological function in modulating host defences against infectious agents (32). Most studies indicate that by effecting the release of appropriate amounts of TNF-α in respond to bacterial infection, mast cells benefit the host in facilitating early bacterial clearance. Additionally, the mast cell products tryptase and histamine can affect the immune system through the recruitment of neutrophils, as is the case for tryptase, or through effects on other immune cells, as is the case for histamine (23). These findings show that mast cells are essential in the initiation and regulation of the innate immune response.
Although the interaction between mast cells and the innate immune system has been clearly established, the potential function of mast cells in the control of *Listeria* infection has so far not been investigated. As documented in this work, the present data do indeed show that mast cells also play a very important role in the initiation of antilisterail innate immune responses.

1.1.3 Endothelial and Epithelial cells

1.1.3.1 Enterocytes

Upon oral *Listeria* infection, a study by Racz et al (33), showed that bacteria resided mostly in the absorptive epithelial cells of the apical area of the villi in the initial stages. In later phases however, the bacteria were found mostly inside macrophages of the stroma of the villi. This early findings already suggested that *L. monocytogenes* penetrates the host by invading the intestinal epithelium (33). This is consistent with the observation that *L. monocytogenes* is able to penetrate the apical surface of polarized, differentiated human enterocyte-like Caco-2 cells with a brush border (34).

In other studies using mice, no invasion of the intestinal villous epithelium was observed; instead, there was colonization of the Peyer's patches (35;36), suggesting that *L. monocytogenes* uses the M-cell epithelium as a port of entry into mice.

1.1.3.2 Hepatocytes

As already mentioned, once it crosses the intestinal barrier, *Listeria* is rapidly trapped from the bloodstream by the liver Kupffer cells which then pass the bacteria on to the hepatocytes. At 6 h after infection, hepatocytes, the preferential targets of *Listeria* infection and replication in the liver, contain more than 90% of the total number of *Listeria* organisms (4;10). Evidence supports the participation of hepatocytes in antibacterial defences during *Listeria* infection in a number of ways, including secretion of immunostimulatory factors, killing of *Listeria*, apoptosis, and interaction with neutrophils. Hepatocytes are also a potentially important source of chemokines for recruitment of effector cell populations (4).

1.1.4 Lymphoid cells

Although the innate immune system is important in the containment of *L. monocytogenes* sterile clearance requires the adapative immune system mediated mainly by T cells. Although antibodies could also be involved (37), their contribution is likely of minor importance.

Natural Killer (NK) cells are large granular lymphocytes that play an important role in the innate host defences. Upon activation, NK cells exhibit lytic activity without restriction by MHC class I molecules and produce cytokines, especially IFN-γ. Highly purified human peripheral blood NK cells cultured with either live or heat killed Listeria lyse NK-cell-sensitive targets and produce IFN-α and TNF-α (38). Thus NK cells may contribute to antilisterial responses directly, by lysing infected cells, and / or indirectly by secreting IFN- α which activate other effector cells.

1.1.4.1 T cells

L. monocytogenes administered by intravenous inoculation is taken up from the bloodstream by splenic and hepatic macrophages. In the spleen, marginal-zone macrophages probably internalise circulating bacteria (13). Histological analysis of spleens from mice infected with *L. monocytogenes* showed that cells containing live,

cytosol-invasive bacteria migrate to the T-cell zones of the splenic white pulp within 24 hours of infection (39). Bacterial transport to the T-cell zone is accompanied by substantial apoptosis in the T-cell compartment, a process that seems to require type I interferons (IFN-β and IFN-α) (40-45). The splenic T-cell zone is where infected macrophages and TipDCs meet. The resulting inflammatory 'battleground', which is surrounded by DCs and T cells, most probably produce antigens that undergo cross-presentation to activate $CD8^+$ T cells. Because non-antigen-specific T cells are speculated to be a principal source of interferons early in infection with *L. monocytogenes* (46), it is possible that the inflammatory process in the splenic T-cell zones drives both the production of IFNs and the subsequent death of T cells.

1.2 Internalization and intracellular life cycle of *L. monocytogenes*

To cause disease, *Listeria* must traverse a series of formidable host barriers such as the intestinal epithelium and the placental and blood-brain barriers. In addition to uptake by phagocytes, *L. monocytogenes* can induce its own uptake by non-phagocytic cells, an aspect that plays an essential role in breaching those natural barriers. Entry into cells involves several sets of bacterial and cell-surface components acting together to mediate adherence and entry. The multisystemic nature of listerial infection indicates that *L. monocytogenes* probably recognizes a number of different eukaryotic receptors. Such receptors include the transmembrane glycoprotein E-cadherin (47), the Met receptor for hepatocyte growth factor (HGF) (48), and components of the extracellular matrix (ECM) such as heparin sulfate proteoglycans (HSPG) (49) and fibronectin (50). The C3bi and C1q complement receptors have been reported to be involved in *L. monocytogenes* uptake by phagocytic cells (51-53). The macrophage scavenger receptor was shown to bind *L. monocytogenes* lipoteichoic acids and hence may also be involved in *Listeria*-macrophage interactions (54).

As mentioned above, the natural route of infection with *L. monocytogenes* is through the gastrointestinal tract. *L. monocytogenes* infects intestinal epithelial cells in a process that requires the interaction of Internalin A (encoded by *InlA*) which is expressed at the cell surface of the bacteria, with epithelial cadherin (E-cadherin), which is expressed at the surface of epithelial cells (55). Although *L. monocytogenes*

has a broad host range, the efficiency with which intestinal epithelial cells from different mammalian species are infected varies. Mice for example, are relatively resistant to intestinal infection with *L. monocytogenes* because of a single amino-acid difference between human and mouse E-cadherin (56;57).

Bacteria traverse the epithelial-cell layer and disseminate in the bloodstream to other organs, such as the spleen and liver, where they are internalised by splenic and hepatic macrophages. In the liver, *L. monocytogenes* enters hepatocytes by expressing another surface protein, Internalin B (encoded by *InlB*) which binds the hepatocyte growth factor (HGF) receptor present at the cell surface of hepatocytes (48).

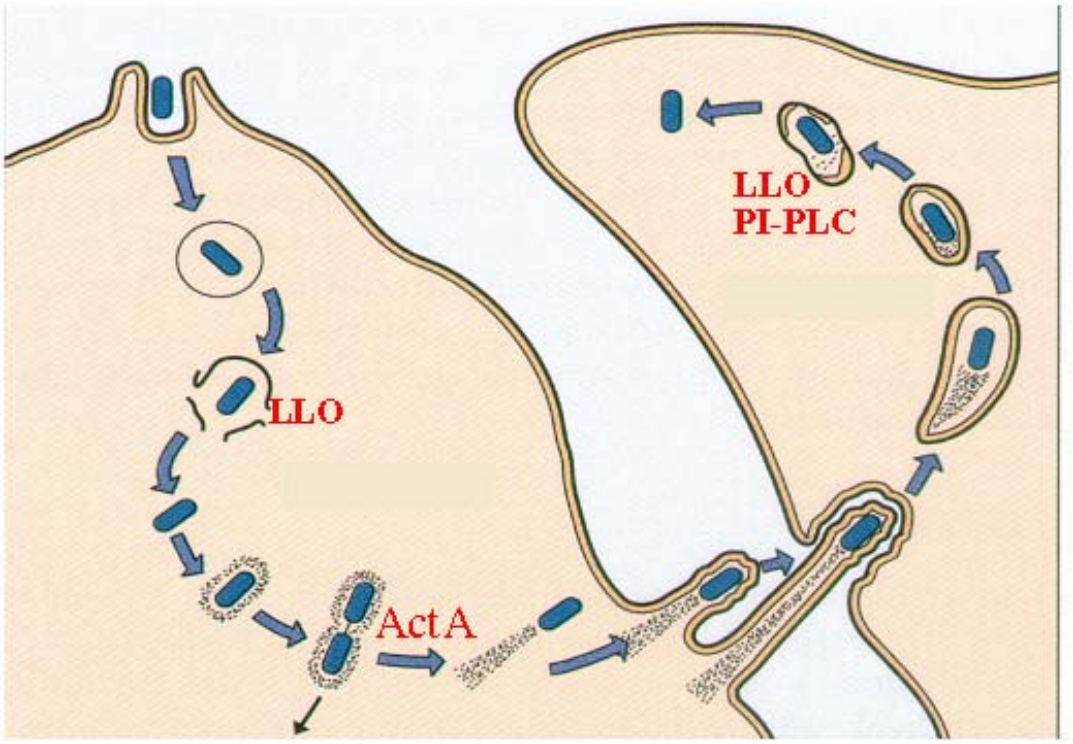

Figure 1.1. Life cycle of *Listeria monocytogenes*.

After cellular invasion, *L. monocytogenes* escapes the phagosome by secreting listeriolysin O (LLO), a virulence factor that destroys the phagosomal membrane (58). In some cell types, such as HeLa cells, escape from the vacuole to the cytosol can be mediated by listerial phospholipases (encoded by *plcA* and *plcB*), independently of LLO (59;60). LLO is to a large extent the main focus of the present work and as

discussed in more details below, LLO besides facilitating vesicular escape, plays multiple roles in pathogenesis of *Listeria.*

Motility of the bacteria in the cytosol is mediated by the surface protein ActA (encoded by *actA*), which nucleates polymerization of actin into polarised actin tails that propel the bacteria towards the membrane where they get enveloped into filopodium-like structures (also called `listeriopods´), which are recognized and engulfed, by adjacent cells. Through the co-operative action of LLO and, the *Listeria* phospholipases the bacteria consequently free themselves from the resultant double-membrane vacuoles into the adjacent cells (33;61;62). Thus, by direct cell-to-cell spread, bacteria disseminate within host tissues while protected from antibodies or complement.

1.3 Role of Listeriolysin in infection

Listeriolysin O (LLO), the pore-forming toxin of *Listeria monocytoneges,* which to a large extent is the main focus of the present study, is considered to be the most important virulence factor of *L. monocytogenes*. LLO is a member of Cholesterol-Dependent-Cytolysins (CDCs), a large family of toxins currently comprising 23 toxins produced by five different genera of Gram-positive bacteria. These toxins commonly bind to cholesterol in the host cell membrane, and share a considerable degree of structural similarity suggesting that they all derive from a common ancestral gene.

Monomers of these toxins bind to cholesterol-containing membranes, in which they oligomerize to form ring or arc-shaped oligomeric complex pores composed of 50-80 subunits. These large pores of at least 20-30 nm in diameter (63-66) disrupt host cell membranes thus allowing the transit of bacteria from one compartment to the other within the cell.

Although breaking membrane barriers to allow cytosolic invasion by *L. monocytogenes* is the original function for which the initial studies were ascribed to, accumulating evidence now indicate that LLO is a multifunctional virulence factor with many important roles in the host-parasite interaction. Exogenous and endogenous exposure to LLO may induce a number of host cell responses, such as cell proliferation and focus formation in transfected fibroblasts (67), activation of the Raf–Mek–mitogen-activated protein (MAP) kinase pathway in epithelial cells (68;69), mucus exocytosis induction in intestinal cells (70), modulation of bacterial

internalisation via calcium signalling (71;72), induction of cytokine expression in macrophages (73;74), degranulation and leukotriene formation in neutrophils (75), induction of apoptosis (42;76), NF-κB activation and expression of cell adhesion molecules in infected endothelial cells (1;77).

The significance of the host responses triggered by LLO is underscored by the fact that mutant strains lacking LLO are not only avirulent (as mentioned above) but that they are also incapable of generating protective immunity in mice. Substitution of LLO with the analogous members of the cholesterol dependent cytolysins (CDCs) such as ivanolysin O (ILO) and an attenuated variant of pefringolysin (PFO) allowed normal phagosomal escape and survival in macrophages *in vitro* but failed to compensate fully for the functions of LLO since such bacteria showed a limited *in vivo* survival. (78; 79). Thus, despite the shared features, LLO possesses unique pathogenic properties that cannot be made up for by the other CDCs Since these toxins can allow efficient phagosomal escape of *Listeria*, the limited *in vivo* survival of such bacterial variants could probably be due an inability to trigger particular host cells responses necessary for survival.

1.3.1 What are the mechanisms of signal induction by LLO?

The mechanisms by which LLO trigger such multiple and diverse cellular responses such as those exemplied above, are still poorly understood. Some of the responses such as mucus exocytosis in intestinal cells, are clearly dependent on the pore forming activity of LLO (70). Recent data suggest that LLO induces calcium signalling in host cells (71;80). Since Ca^{2+} oscillation modulates several signalling processes, the data may in part explain the broad spectrum of cellular responses induced by LLO. However, pore formation *per se* does not explain it all. Inhibition of pore formation by pre-incubation of LLO with cholesterol does not affect its ability to bind and induce particular signals in target host cells (66;81;82). This implies that LLO has multiple biological activities and can trigger signalling via mechanisms that are independent of pore formation.

To understand the pore dependent and pore independent mechanisms, induction of calcium fluxes by LLO and the role of the cholesterol rich membrane microdomains (lipid rafts) have been investigated in this work. Section 1.4 gives a brief introduction into calcium signalling by bacterial pathogens components while section 1.5 give a

general introduction into lipid rafts and their role in signal transduction and in host-pathogen interaction.

In addition to the signals triggered by LLO, *L. monocytogenes* can also trigger signals independently of LLO. To investigate these mechanisms, the role of toll-like receptors (TLRs), in the induction of proinflammatory signals by *L. monocytogenes* was also investigated. Section 1.6 gives a general introduction into signal transduction via these receptors.

1.4 Induction of calcium signalling in host cells during interaction with bacterial pathogens

Ca^{2+} is a ubiquitous intracellular messenger, controlling a diverse range of cellular processes, such as metabolism, gene transcription, cell proliferation, fertilization, muscle contraction, apoptosis, exocytosis and cytoskeletal reorganization (83). In addition, induction of cellular calcium flux has emerged as a widespread mechanism by which many pathogenic bacteria influence host cells. Among the mentioned Ca^{2+} dependent processes, alteration of the host`s metabolism, induction of apoptosis and the control of gene expression, especially that leading to the expression and secretion of proinflammatory mediators as well as cytoskeletal reorganization, are of particular significance during bacterial infection. More and more studies now show that the mechanisms of Ca^{2+} signal induction are as multiple and diverse as the virulence factors in question. The list in Table 1 shows a few examples of bacterial pathogens and the respective virulence factors responsible for calcium signalling in host cells.

Because LLO is of particular relevance in the present studies, this introduction will dwell more on induction of Ca^{2+} fluxes by pore forming toxins.

Ca^{2+} signalling by pore forming toxins has been ascribed to the influx of extracellular Ca^{2+} as well as the release from intracellular Ca^{2+} stores (80;84). In most cases, induction of Ca^{2+} influx by pore-forming toxins occurs largely due to the passive diffusion of ions via the toxin transmembrane pores. This is especially true for the large non-selective pores formed by the Cholesterol Dependent Cytolysins (CDCs) such as LLO. Because of their large size, such pores can allow the passage of ions and macromolecules into and out of the cell (85). In addition to passive diffusion, calcium signalling by some pore forming toxins has been shown to involve the opening of membrane Ca^{2+} channels.

Ca^{2+} influx as a result of the opening of Ca^{2+} channels by a pore-forming toxin has best been demonstrated for the staphylococcal leukotoxins. It is thought that the leukotoxins bind to either a receptor linked to a divalent cation-selective channel, or to the channel itself thus activating it. The leukotoxins then open a second pathway by insertion into the membrane and subsequent formation of aspecific pores allowing influx of other molecules (86).

Organism	**Virulence factor**	**Mechanism**
Escherichia coli	α-hemolysin (HlyA)	Ca^{2+} influx and release from intracellular stores
	Heat-labile enterotoxin (Etx)	Ca^{2+} influx
	Heat-stable enterotoxin B (STB)	Ca^{2+} influx
	Heat-stable enterotoxins A (STa)	Release from intracellular Ca^{2+}stores
	Cytolysin A	Ca^{2+} influx and release from intracellular stores
Vibrio cholerae	Non-agglutinable Sta (NAG-ST)	Release from intracellular Ca^{2+}stores
	Cholera toxin B	Ca^{2+} influx
Bacillus anthracis	Anthrax edema factor (EF)	Ca^{2+} influx
Clostridium difficile	Toxin B	Ca^{2+} influx
Clostridium perfringens	Enterotoxin, α-toxin	Ca^{2+} influx
	α-toxin and Perfringolysisn O	Ca^{2+} influx
Shigella flexneri	Type III effectors	Release from intracellular stores
Salmonella enteritidis	Type III effectors	Release from intracellular stores
	FliC flagellin	Release from intracellular stores
Pseudomonas aeruginosa	Type III effectors(ExoU) and type IV pili	Release from intracellular stores
	FliC flagellin	From intracellular stores
Staphylococcus aureus	Staphylococcal α-toxin	Ca^{2+} influx and from intracellular stores,
	Bicomponent leukotoxins of γ-hemolysins(HlgA, HlgB, HlgC)	Ca^{2+} influx
Aeromonas hydrophila	aerolysin	Ca^{2+} influx and release from intracellular stores
Neisseria gonorrhoeae	Membrane porin (PorB)	Ca^{2+} influx
Yersinia enterocolitica	Heat stable enterotoxin (Y-STa)	Ca^{2+} influx
	γ-Phospholipase C	Release from intracellular stores
Mannheimia haemolytica	Leukotoxin (LKT)	Ca^{2+} influx and release from intracellular stores
Group A Streptococcus (GAS)	Streptolysin O (SLO)	Ca^{2+} influx
Listeria monocytogenes	Listeriolysin O	Ca^{2+} influx
	Posphatidylinositol phospholipase C (PI-PLC)	Release from intracellular stores

Table 1. Bacterial pathogens and virulence factors that induce calcium signalling in host cells. Compiled from TranVan et al (84)

A common feature in Ca^{2+} signalling by Gram-negative bacteria such as *Pseudomonas aeruginosa, Shigella flexineri, Salmonella enteritidis*, and *Helicobacter pylori* is that Ca^{2+} signalling is dependent on the delivery of the effector via the type III and / or IV apparatus (84). Since the targeting of effectors into the host cell cytosol by the type III apparatus depends on the formation of a pore within host cell membranes, the mechanism underlying the elevation of cytosolic Ca^{2+} by this mechanism presents a close similarity with some pore forming toxins such as the CDCs. In fact, based on several recent studies, CDCs are now considered to be the equivalent of type III secretion apparatus in Gram-positive bacteria for delivering molecules into the host cell (87). In that respect, LLO has been found to play the accessory role of delivering PI-PLC - a *L. monocytogenes* phospholipase, into the cytosol where it ultimately triggers the release of Ca^{2+} from intracellular stores via the generation of Inositol triphosphate (IP_3) (88). Thus, LLO plays at least two roles in the induction of Ca^{2+} signals; (1) directly inducing the influx of Ca^{2+} (71;88), (2) modulating Ca^{2+} release from intracellular stores by allowing PI-PLC access to the host´s phosphatidylinostitol diphosphates.

As illustrated, the fact that several bacterial components participate in the induction or modulation of these responses presents a level of complexity in delineating the mechanisms underlying bacteria-induced Ca^{2+} signalling. This is further complicated by the fact that Ca^{2+} signal induction even by a single bacterial component, could involve multiple signalling pathways. Therefore, despite the recent advances, the precise mechanisms underlying the induction of Ca^{2+} responses by LLO, let alone *L. monocytogenes*, remain by and large uncharacterized.

1.5 Role of lipid rafts in the interaction of pathogens with hosts cells

The “fluid mosaic” theory originally proposed by Singer and Nicholson (89) is still the text book model that explains the architecture of the plasma membrane. According to this model, the plasma membrane is a two-dimensional lipid solvent in which packing is loose and lateral diffusion is relatively rapid. The implications of such a model is that all plasma membrane proteins are uniformly dispersed in the lipid solvent, akin to `icebergs in a sea of lipids. In the past two decades however, many independent investigators have acknowledged that the plasma membrane is not as homogeneous as proposed (90). Biophycisists find that lipids in the bilayer model exist in several phases ranging from the quasi-solid gel phase on one extreme, to the fluid mosaic or fluid-crystalline (I_c) phase on the other. In the quasi-solid gel phase, phospholipids with saturated hydrocarbon chains such as sphingolipids (glycosphingolipids and sphingomyelins) pack tightly with cholesterol to form lateral assemblies within the bilayer onto which specific proteins attach. These assemblies are now generally referred to as membrane microdomains. In resemblance to `vessels floating in a sea of lipids´, the analogous term lipid rafts has recently been adapted to refer to such microdomains.

1.5.1 Conception of the raft hypothesis

The turning point in the way the plasma membrane structure is viewed came with the conception of the raft hypothesis (91-95). It was formulated based on studies carried out on epithelial cell polarity (96). In epithelial cells, plasma membranes are distinct into apical and basolateral domains, with the former being enriched in sphingolipids and the latter in glycerolipid phosphatidylcholine (97). Proteins with glycosylphospatidyl inositol (GPI) anchors were also shown to be expressed exclusively on the apical surface of several epithelial cell lines (98). Studies on the delivery of newly synthesised lipids in such cells revealed that a simple glycosphingolipid was preferentially transported to the apical membrane (97;99;100). To explain this preferential delivery, Simons and colleagues (99) proposed that glycosphingolipid-rich microdomains that also contain proteins, form within the

exoplasmic leaflet of the Golgi apparatus and become packaged into vesicles for apical transportation.

The initial direct experimental support for the raft model came from the behaviour of GPI-anchored proteins (101). These proteins were shown to partition into detergent-resistant membranes (DRMs) enriched in glycosphingolipids with the exclusion of basolateral marker proteins. In addition, partitioning of these proteins into DRMs occurred in the Golgi complex, thus implying that protein-sphingolipid microdomains form in the Golgi apparatus and are exported to the plasma membrane. As described in the next sections, these microdomains may also be found in other vesicles. Cumulatively, these studies suggested that GPI anchors and association with the DRMs could target proteins to the apical membranes. However, in the meantime, it is clear that other membrane microdomain (raft) targeting signals exist (see below).

Although scientists are now just beginning to unravel the dynamics of the Glycosphingolipid–protein microdomains (lipid rafts), caveolae - small invaginations of a similar glycolipid composition, found on the surface of many cell types, had already been discovered by Palade and Yamada in the 1950s. As will be covered in more detail below, caveolae share many features with the classical lipid rafts. For instance, it was shown that caveolin (the main structural protein of caveolae) co-purified with DRMs and is also present in the post-Golgi transport vesicles (102). One outstanding question that emerged from these observations was: what functional attributes besides the shared glycolipid compositions, could the two share? Before any precise function was ascribed to the lipid rafts, suggestive evidence to the effect that caveolae may be involved in endocytosis (103), transcytosis (104) and signalling (98;105) was already in existence. Therefore, given these similarities with caveolae in terms of biogenesis and the glycolipid compositions, the putative functions of rafts, was simply a consequence of logical association. Assimilation of all the above lead to the formulation of the raft model (Figure 1.2) proposed by Simons and colleagues (94). Although it is still under intense debate (106;107), this model is the current unifying concept on the dynamic organizational structure and function of the lipid bilayer.

According to this model, sphingolipids associate with one another, probably by weak interactions between their carbohydrate heads. The sphingolipid head groups occupy larger excluded areas in the plane of the exoplasmic leaflet than do their predominantly saturated lipid hydrocarbon chains.

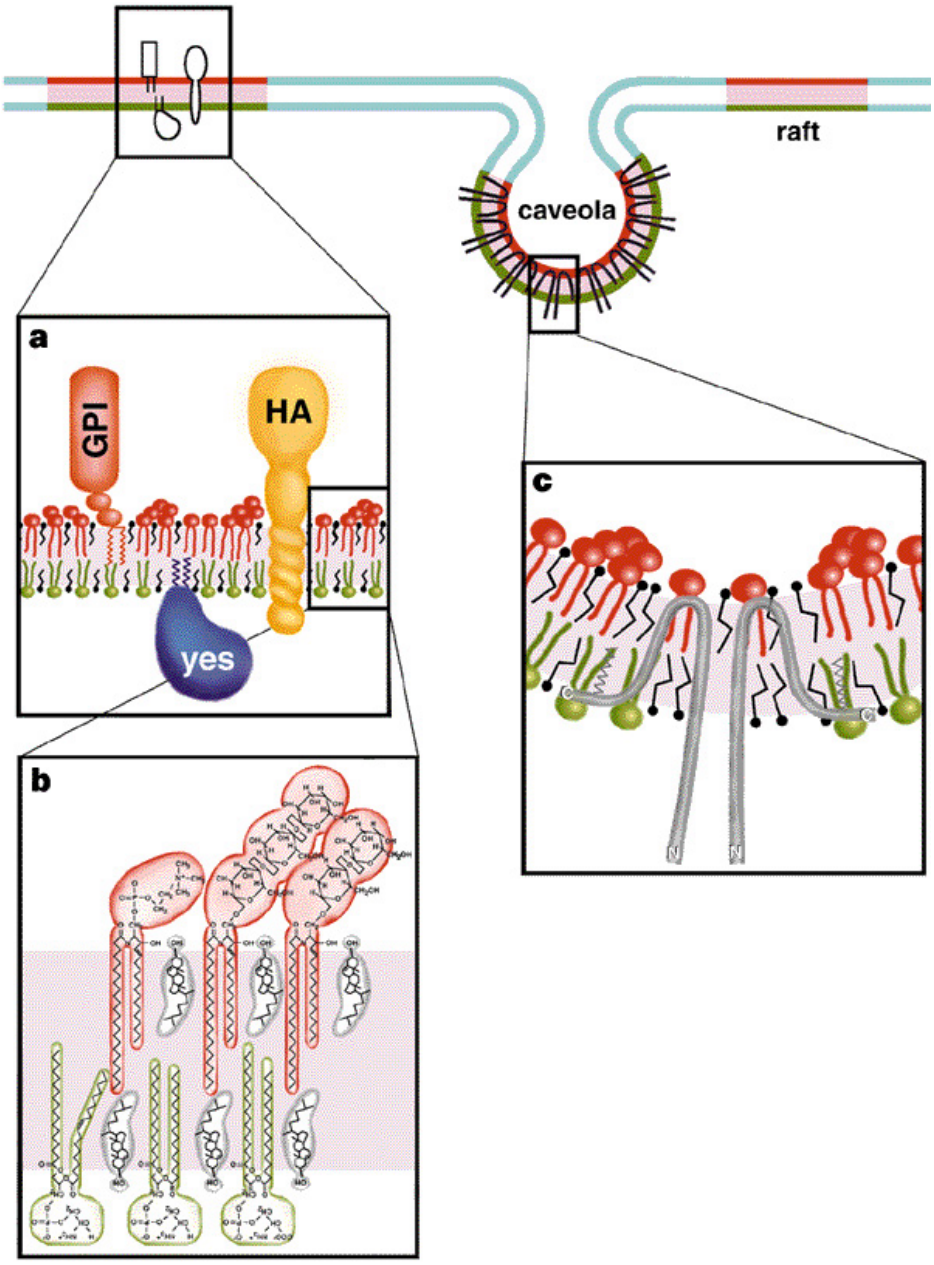

Figure 1.2. Diagram Summarizing the Membrane Organization of Lipid raft and Caveolae. The rafts (red) segregate from the other regions (blue) of the bilayer in the exoplasmic leaflet. The blue region (predominantly enriched in phosphatidylcholine) has a different organization from that of the rafts. (**a**) Rafts contain proteins attached to the exoplasmic leaflet of the bilayer by their GPI anchors. Acylated proteins (the Src-family kinase Yes) are shown binding to the cytoplasmic leaflet, while the influenza virus proteins neuraminidase and haemagglutinin (HA) associate with rafts through their transmembrane domains. (**b**) The lipid bilayer in rafts is asymmetric, with sphingomyelin (red) and glycosphingolipids (red) enriched in the exoplasmic leaflet. Glycerolipids such as phosphatidylserine and phosphatidylethanolamine (green) are enriched in the cytoplasmic leaflet while cholesterol (grey) is present in both leaflets and fills the space under the head groups of sphingolipids or extends the interdigitating fatty acyl chain in the opposing leaflet. (**c**) Caveolae are formed by self-associating caveolin molecules forming a hairpin loop in the membrane. Interactions with raft lipids may be mediated by binding to cholesterol and by acylation of C-terminal cysteines. (94)

Any voids between associating sphingolipids are filled by cholesterol molecules which function as spacers (Figure 1.2b). The close-packed sphingolipid–cholesterol clusters behave as assemblies within the exoplasmic leaflet, whereas the intervening fluid regions are occupied by unsaturated phosphatidylcholine molecules (Figure 1.2a). Glycosphingolipids that carry long fatty acids which are amide-bonded to the sphingosine base interdigitate with the cytoplasmic leaflet of the bilayer. Since cholesterol is present in both leaflets, it was suggested that it functions as a spacer in the cytoplasmic leaflet as well, filling voids created by interdigitating fatty acid chains (Figure 1.2b).

Although the formation of membrane microdomains in the exoplasmic leaflet can be explained by phase separation of sphingolipids and cholesterol, the lipid composition and the organization in the corresponding cytoplasmic leaflet has not been defined. Besides cholesterol, it probably also carry mainly saturated fatty acid chains to optimise packing. Individual lipids may also move in and out of the rafts, explaining why sphingolipid–cholesterol clustering is difficult to detect spectroscopically.

1.5.2 Physico –chemical properties of rafts

One reason why it has been difficult to prove that rafts exist in cells is their size. By use of flourescence resonance energy transfer (FRET), the size of individual rafts has been estimated to be smaller than 10 nm in diameter (108-110). This size precludes detection by standard light microscopy. However, if rafts are cross-linked with antibodies or lectins in living cells, they then cluster together in such a manner that raft and non-raft components separate into micron-sized quilt-like patches that can be visualized microscopically (111;112). Oddly, the strongest experimental support for the existence of rafts came from seemingly unrelated findings, namely their high melting temperature (Tm) and insolubility in non-ionic detergents (113). Plasma membranes are partially resistant to solubilization by non ionic detergents like Triton X-100 in the cold (113) or like Polyoxyethylene ether at physiological temperatures (114). Membrane fragments extracted with these detergents float to low density during sucrose gradient centrifugation and are enriched in raft associated proteins and lipids. This has provided a simple means of identifying possible raft components. As a consequence, rafts are also referred to as: Detergent-Resistant Membranes (DRMs), Detergent-Insoluble Glycolipid-rich domains (DIG), Detergent–Insoluble Membranes (DIM) and Low-density Triton-insoluble (LDTI) complexes.

Despite the ease and usefulness of non-ionic detergent extraction, this method is not without its shortcomings. For instance, milder detergents such as octylglucoside, will solubilize lipid rafts (115). Another problem with detergent extraction is that the original subcellular locations of DRMs are unknown. Moreover, association of a protein with the raft may be so weak that it is solubilized by the detergent. Association of some raft proteins with the cytoskeleton can also preclude their detection in the DRMs since they do not float after detergent extraction. Changes in detergents and extraction conditions have also been shown to produce contrasting results (116;117).

1.5.3 Criteria for association of proteins with rafts

One of the most important properties of lipid rafts is that they can, constitutively or inducibly, include or exclude proteins to variable extents. Many of the associated proteins are linked to saturated acyl chains, which is likely to make them prefer an ordered environment. Proteins can be linked to saturated acyl chains in two ways; either in the form of a GPI anchor (118;119) or through myristylation and palmitoylation. For GPI-anchored proteins, the GPI anchors, usually two, determines raft association (120). The importance of acylation in the preferential targeting to rafts has been shown most clearly for the Src family nonreceptor protein tyrosine kinases (121;122). With a few exceptions (123), most groups have found that association of these kinases with rafts require dual modification by both myristate and palmitate (122;124) Based on these findings, acylation is considered to be a raft targeting signal for a wide range of proteins. Though GPI-anchorage and acylation are the only known signals for raft targeting, other signals must exist as some raft proteins contain neither modifications (113). For instance, although most transmembrane proteins generally appear to be excluded from lipid rafts, some do show rafts association. Most of the notable exceptions include, a fraction of linker of activated T cells (LAT) (125), CD4 and CD8 in T cells, integrins in myeloid cells, CD44, and CD26 in lymphocytes, CD36 in platelets (126) and the influenza virus proteins neuraminidase (NA) and haemagglutinin (HA).

1.5.4 Caveolae

Different classes of rafts with specific protein composition have been shown to exist in mammalian cells (114). For instance, caveolae are one distinct subset of rafts.

These flask-shaped plasma membrane invaginations are formed from classical sphingolipid rafts by polymerisation of caveolin – hairpin-like palmitoylated integral membrane proteins. When caveolin is integrated into the microenvironment of a lipid raft, these microdomains invaginate and form caveolae, i.e. 50-100 nm flask-shaped structures located at or near the plasma membrane. Originally, caveolae were believed to be found only in non-hematopoietic cells. However, in recent years, this notion is proving to be null and void because, (i) caveolae have been found to be pleiomorphic with vesicular, flat or tubular forms also existing within cells (127); (ii) caveolins have been found in raft entities in virtually all cell types examined, including hematopoietic cells such as macrophages, dendritic cells and mast cells (128). Thus, caveolae are pleomorphic lateral assemblies of a distinct group of molecules including cholesterol, a 22-kDa protein caveolin-1 and various glycolipids and GPI anchored proteins (129). In recent years caveolae have been implicated in several cellular processes including vesicular trafficking, and signal transduction (130).

1.5.5 Cellular and subcellular distribution of rafts

DRMs have been isolated from almost all mammalian cell types. Immature oligodendrocytes (131) and immature hippocampal neurons are exceptions in that they are poor sources of DRMs. However, as hippocampal neurons mature, their sphingomyelin content and therefore their ability to produce DRMs, increases (113). DRMs have not been well identified in other eukaryotes, although they have been isolated from *Sacharomyces cerevisiae* (132).

The distribution of lipid rafts over the cell surface depends on the cell type. In polarised epithelial cells and neurons, lipid rafts accumulate in the apical and axonal membranes respectively. Basolateral and somato-dendritic membranes also contain rafts, but in smaller amounts. In contrast, caveolae are mainly present on the basolateral side of epithelial cells (133) facing the blood supply. The functional significance as well as the underlying mechanisms of this aberrant distribution is still a puzzle. In lymphocytes and fibroblasts, rafts are distributed over the cell surface without any obvious polarity (110).

The lipid raft dogma was drafted largely based on the morphological characterisation of the sphingolipid-cholesterol assemblies concentrated in the exoplasmic leaflet of the plasma membrane. Nonetheless, it is generally assumed that rafts exist in both the external and internal leaflets of the membrane, and that they overlap so that they

are coupled functionally and structurally. The two monolayers of the plasma membrane of eukaryotic cells have different chemical compositions. This out-of-equilibrium situation is maintained by the activity of lipid translocases, which compensate for the slow spontaneous transverse diffusion of lipids. Thus, although linked, rafts in the outer leaflet, corresponding to domains enriched in sphingomyelin and cholesterol, cannot be identical to those in the inner cytoplasmic leaflet. Brown & colleagues (134) did however observe that some glycerophospholipids may also participate in raft formation, a property that could be critical in the formation of rafts in the inner leaflet. Intimations that rafts also occur in the cytoplasmic leaflet have been drawn indirectly. For example, the fact that Src-family kinases are present in DRMs, although their acyl chains have access only to the inner leaflet, suggests that rafts exist in the cytoplasmic leaflet (113). Similarly, by using a cyan-fluorescent protein (CFP) targeted to inner plasma membrane rafts of Jurkat T cells, Gri and co-workers have found that raft domains at the outer and inner leaflets are physically coupled and that this coupling requires cholesterol (135).

Biological rafts are in general of nm scale, and almost certainly differ in size and stability in inner and outer monolayers. Any coupling between rafts in the two leaflets, should it occur, is considered to be transient and dependent upon not only the properties of lipids, but on transmembrane proteins within the rafts as well (136).

Although lipid rafts are localized mainly at the level of the plasma membrane, they can also form within integral membrane compartments, such as the Golgi apparatus (137) and endocytic vesicles. As noted earlier, the biogenesis of rafts is initiated at the Golgi apparatus. The assembly of the raft proteins with sphingolipid-cholesterol microdomais first occurs at this level before moving to the plasma membrane (101). However, lipid raft trafficking does not end with cell surface delivery. Rafts have been shown to be endocytosed continuously from the plasma membrane (95). From early endosomes, rafts recycle either directly back to the cell surface or return indirectly through recycling endosomes, which could also deliver them to the Golgi (138;139).

1.5.6 Lipid rafts in signal transduction

The most important role of rafts at the cell surface may be their function in signal transduction. One way to consider signalling through rafts is that rafts form platforms that concentrate or separate specific proteins in a unique signalling entity. The best evidence for the involvement of rafts in signalling came from studies on FcεRI, the

receptor for IgE on basophils and mast cells. IgE binds constitutively to cell-surface FcεRI. When antigens are recognised by the bound IgE, FcεRIs are induced to aggregate. Aggregation of these receptors activates the associated Src-family kinase Lyn, initiating a signalling cascade that culminates in degranulation. Studies by Holowka and co-workers showed that aggregation of the FcεRIs led to its recruitment into DRMs to be phosphorylated by Lyn. This was interfered with by depletion of surface cholesterol. (140; 141). These and many other subsequent findings have provided the strongest evidences to date that rafts do indeed form under physiological conditions. Apart from the FcεRI, rafts have also been shown to be involved in several other signalling processes. These include, the T-cell receptor (TCR), B-cell receptor (BCR), epidermal growth factor (EGF), insulin receptor, intergrins, Ephrin and EGF receptors, the H-Ras, glial-cell derived neurotrophic factor (GDNF), endothelial nitric oxygen synthase (eNOS) and the Hedgehog signalling pathways (95;114;142-146) just to mention a few. Since Mast cells are of particular relevance to the present study signal transduction in this cell type has been given more detailed coverage below.

How do lipid rafts regulate signalling? Although appealing working hypotheses have been put forward, there are still unanswered questions on how receptors signal through rafts. For instance, it has been observed that clustering or ligation of cell-surface GPI-anchored proteins can trigger the activation of Src-family kinases in the cytoplasm (95;147;148). Since neither protein penetrate the bilayer, how GPI-anchored proteins send information across the membrane still remains a mystery. A plausible scenario is that GPI-anchored proteins may bind to elements *cis* to transmembrane proteins, thus allowing the transmission of signals across the membrane. Another possibility is based on the observation that GPI-anchored proteins associate with glycosphingolipids. Since glycosphingolipids carry long fatty acid chains that can interdigitate with the cytoplasmic leaflet, it is reasonable to imagine that GPI anchored proteins may exploit them to engage in an indirect communication with the signalling components in the cytoplasmic leaflet. The uncertainties on the precise mechanisms of cytoplasmic /exoplasmic raft interactions notwithstanding, a number of models (Figure 1.3) to explain how extracellular signals are transmitted through rafts have been put forward by Simons K & Toomre (95).

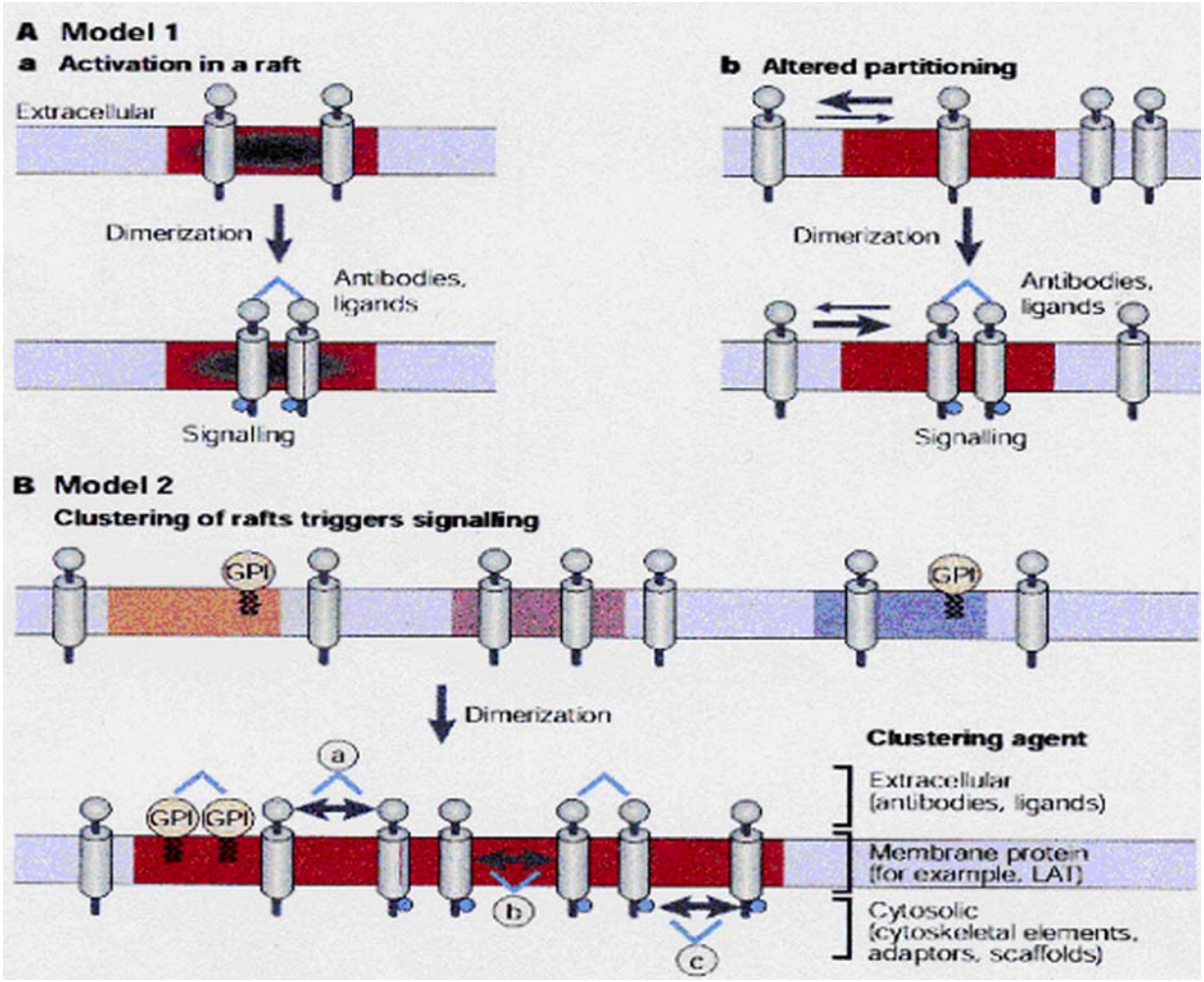

Figure 1.3. Model 1: How signalling can be initiated in single rafts. Receptors associated with rafts at steady state could be induced to aggregate upon ligand binding thus leading to activation of signals (Fig 1.3**Aa**), Alternatively, individual receptors with weak affinity for rafts could oligomerize on ligand binding and this would lead to an increase in residency time in rafts (Fig 1.3**Ab**). **Model 2: How raft clustering induces signalling.** Activated receptors could recruit cross-linking proteins that associate with other rafts, resulting in rafts coalescence (Fig 1.3**B**). Through formation of a raft cluster, a network of interactions between adaptors, scaffolds and anchoring proteins would be built up to organize the signalling complex in space and time. This signalling complex would be insulated within raft clusters from the surrounding liquid-crystalline lipid matrix (I_{ce}). The formation of clustered rafts would lead to amplification through the concentration of signalling molecules, as well as exclusion of unwanted modulators.

The TCR is one of the classic examples of how rafts aggregation induces signalling. This receptor is only weakly associated with detergent resistant membranes (Drums), both in non-stimulated Jurket T cells and in thymocytes (125;149). Following TCR engagement, its association with DRMs increases. This DRM-association correlates with the phosphorylation of immunoreceptor tyrosine based activation motives (ITAMs) in the TCR-ζ chains. Phosphorylation of ITAMs sparks a chain of

phosphorylation events that lead to the recruitment and exclusion of particular proteins. This raft/non raft partitioning initiates the formation of the “immunological synapse” (IS) in which negative modulators such as CD45, and CD43 are excluded (150;151).

The interactions that drive raft assembly are dynamic and reversible. Raft clusters can be disassembled by negative modulators and /or by removal of raft components from the cell surface by endocytosis (95). The size of rafts in relation to signalling is still a hotly debated issue. For instance, whereas there are strong experimental data to argue for the aggregation of rafts into large assemblies, there are counter arguments why domains should be much smaller in biological membranes. To have very large rafts would seem to negate the effectiveness of rafts as a dispersed, regulatory structure. In natural membranes therefore, aggregation and/or disassembly of rafts could be under regulatory factors such as the cytoskeleton to provide sizable domains with appropriate functionality (152).

1.5.7 Role of rafts / caveolae in infection

Since the discovery of lipid rafts less than two decades ago, scientists are now coming to terms with the realisation that after all, these structures were already discovered millions of years ago by microbes. During these years, pathogenic microbes have evolved a myriad of strategies for communication and coexistence with their target host. The importance of rafts in that respect is highlighted by their enrichment in signalling molecules that make them a natural target for microbes to communicate with the host. Lipid rafts are also known to undergo fission from the plasma membrane, mediating a form of endocytosis that is different from clathrin-coated pit internalisation (153). Hence, microbial agents might also favour interaction with lipid rafts as a potential way to enter host cells.

To evade immunological defences and establish a secure `niche´ within their hosts, pathogens employ several mechanisms of avoidance. Some avoid ingestion by the phagocytic cells designed to degrade them. Others promote their uptake and reside within `safe´ compartments inside host cells, protected from cellular mechanisms of microbial degradation.

Classic endocytosis depends on clathrin coated pits and involves an intracellular pathway in which lysosomes fuse with internalised vesicles, degrading their contents (154). In contrast, and in clear benefit to pathogens, raft/caveolae-dependent

endocytosis does not feed into the lysosome pathway and does not result in the degradation of the cargo in the raft endocytic vesicles (128;154). If taken up by classic endocytosis, intracellular pathogens must avoid degradation in the endosome-lysosome pathway, either by escaping from endocytic vacuoles (phagosomes) into the cytoplasm before lysosomes fuse with phagosomes or by actively neutralizing microbicidal agents inside the phagolysosome after fusion (128;154). `Smart´ pathogens and bacterial toxins can avoid this problem by binding to caveolae/rafts and triggering endocytosis through a pathway that avoids lysosomes altogether. A wide range of pathogens (or their products) including viruses, parasites, bacteria and their toxins have been suggested to co-opt rafts for their benefit (refer to table1 and reviews (154;155;156).

1.5.7.1 Lipid rafts in the internalisation of *L. monocytogenes* and other pathogens

As mentioned above, a wide range of pathogens prefer raft-like ordered membrane domains for entry into the cell. For instance, *E. coli* strains that express FimH (bacterial adhesin) can gain entry and survive in phagoytic cells via CD48, a GPI-anchored protein present in the rafts (91). Internalisation of *Mycobacteria* bovis has also been shown to be cholesterol dependent (92), suggesting an involvement of rafts in the uptake. Interestingly, the uptake of *Plasmodium falciparum* by erythrocytes, a cell type that is normally incapable of endocytosis /phagocytosis, seems to be mediated by rafts (155).

Certain viruses also preferentially interact with rafts at sites for internalisation or for budding. Entry via rafts/caveolae has especially been shown for SV40. SV40 binds to MHC class I molecules and by an unknown mechanism leads, to the recruitment of caveolae around the virus whereby generation of a caveolae-like vesicle delivers the virus into the endoplasmic reticulum. It has also been proposed that the initial binding of HIV to CD4 promotes raft clustering (155).

Pathogens	Receptors in rafts	Function	References
Viruses			
HIV	CD4, CXCR4, CCR5	Budding/viral fusion/trancytosis	(156; 157)
Ebola and Marbug viruses		Entry/Budding	(158; 159)
Measles virus		Assembly and budding	(156; 159)
simian virus 40 (SV40)		Caveolae-mediated entry	(160)
Influenza virus		Budding	(161)
Respiratory syncytial virus (RSV)		Replication	(162; 163)
Herpes simplex virus		Entry/Budding	(164; 165)
Epstein Barr virus		Signalling	(156; 166)
Echovirus 1		Entry	(167)
Echovirus 11		Entry/budding	(168)
Enterovirus 70	CD55/DAF	Entry	(169)
Human T cell leukaemia virus type 1		Entry/Budding	(170)
Semliki-forest virus		Entry	(171)
Bacteria			
FimH-expressing *E.coli*	CD48	Entry/signalling	(154; 172)
Shigella flexineri	CD44	Entry/Signalling Cytoskeletal rearrangement	(173)
Campylobacter jejuni	Cholesterol	Entry	(156)
Salmonella typhimurium	EGF receptor	Entry	(155)
Mycobacterium spp	Cholesterol	Entry/ intracellular survival	(174; 175)
Brucella spp.	GM1, cholesterol, Scavenger receptor	Entry/ intracellular survival	(176; 177)
Pseudomonas aeruginosa		Signalling	(178)
Legionella pneumophila		Entry	(179)
L. monocytogenes	E-cadherin / HGF receptor	Entry	(180)
Toxins			
Lipopolysaccharide	CD14	Signalling	(181)
E. coli Heat-labile enterotoxins	GD1	Entry, signalling	(182)
Aeromonas hydrophila toxin (Aerolysin)	GPI-anchored proteins	Binding and oligomerization	(183)
Clostridium perfringens toxin (Perfringolysin O)	Cholesterol	Oligomerization, signalling	(184)
L. monocytogenes toxin (Listeriolyisn O)	Cholesterol	Oligomerization, signalling	(112;185)
Eisenia foetida protein (Lysenin)	Sphingomyelin		(186)
Vibrio cholera cytolysin	Cholesterol	Oligomerization	(155)
Cholera toxin	GM1	Binding, signalling	(155)
Helicobacter pylori toxin (VacA toxin)	GPI-anchored proteins	Signalling	(187)
Parasites			
Toxoplasma gondii		Intracellular survival	(188)
Plasmodium falciparum		Entry	(189)
Theileria parva		Signalling	(190)

Table. 2. Pathogens and virulent factors that interact with host cells via rafts**.**

There is also growing evidence to support the hypothesis that pathogenesis of prion disease is localized to caveolae-like domains. A region of the GPI-linked cellular prion protein targets it specifically to caveolae. This is an essential step in the conversion of the prion protein into the pathogenic form (93).

Recent studies by Cossart and co-workers suggest that *L. monocytogenes* the pathogen of interest in the present study also gains entry into nonphagocytic cells via rafts. Internalisation was found to be depended on membrane cholesterol and the presence of bacteria receptor E-cadherin in the DRM (180). Since the basolateral side of the epithelia (entry side of *Listeria*) is largely devoid of rafts associated molecules as described in section 1.5.5, the above findings are quite controversial. Furthermore, another study has reported that both the receptors for *L. monocytogenes,* c-met and E-cadherin predominantly reside in non-DRM fractions (191). Thus, the role of lipid rafts in the uptake of *L. monocytogenes* remains unclear.

1.5.7.2 Rafts as concentration platforms for toxins

Pore forming toxins utilize the concentration capacity of rafts not just for initial binding, but also rather for the subsequent oligomerization step which is a prerequisite for channel formation. This has been shown for aerolysin (*Aeromonas hydrophila* toxin) which specifically binds to GPI-anchored proteins and a variety of other pore forming toxins, including streptolysin O, perfingolysin O (PFO), lysenin, tetanus toxin, cholera toxin, *Vibrio cholera* cytolysin and *Clostridium* alpha toxin (155;183;184;192) (Table 1).

1.5.7.3 Rafts as signalling complexes for pathogens

While some pathogens exploit lipid rafts for entry, the high concentration of receptors and signalling molecules in these domains qualifies them as ´detonation centres` for pathogens to initiate an inflammatory response by the host. For instance, engagement of CD48 by bacterial FimH triggers secretion of the pro-inflammatory cytokine tumor necrosis factor–α (TNF-α) (193). Lipopolysaccharide (LPS), a potent toxin produced by all Gram-negative bacteria, also interacts with rafts via the GPI anchored CD14 to trigger the MAP kinase pathway and cytokine production (155).
This illustrates the importance of lipid rafts in cellular functions and how pathogens communicate with their target host cells via these membrane microdomains.

1.6 Pathogen Recognition by Toll-like receptors

Toll receptor was originally identified in *Drosophila* as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos (194). In 1996, Hoffmann and colleagues demonstrated that Toll-mutant flies were highly susceptible to fungal infection (195), a study that higlighted that the immune system, particularly the innate immune system, has a skillful means of detecting invasion by microorganisms. Subsequently, mammalian homologues of Toll receptor were identified one after another, and designated as Toll-like receptors (TLRs) (196). Over the last few years, it has become evident that members of the TLR family mainly transfer both the recognition and the subsequent response to pathogens. Eleven TLRs (TLR1-11) have been described so far (197-199).

The cytoplasmic portion of TLRs shows high similarity to that of the interleukin (IL)-1 receptor family, and is now called the Toll/IL-1 receptor (TIR) domain. Despite of this similarity, the extracellular portions of both types of receptors are structurally unrelated. The IL-1 receptors possess an Ig-like domain, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain. Each TLR has been shown to recognise specific pathogen-associated molecular patterns (PAMPs) (200), i.e conserved microbial patterns shared by large groups of pathogens, but not found in higher eukaryotes except under pathological conditions such as stress (197-199). Of the eleven TLRs described so far, TLR2 and TLR4 are the most relevant in the present studies and the best-investigated family members. TLR4, the Lipopolysacharide (LPS) receptor, is important for the recognition of Gram-negative bacteria, whereas TLR2 has been designated the major receptor for Gram-positive bacteria by virtue of its capacity to recognize major cell wall constituents of Gram-positive microorganisms, such as peptidoglycan (PGN), lipoteichoic acid (LTA), and lipoproteins (197-199). TLR2 homodimers or, TLR1/TLR2 and TLR2/TLR6 heterodimers, have been demonstrated to migrate to phagosomes within phagocytic cells, where they might sample the contents and signal the presence of an invader (201;202). Heterodimerization of TLR2 with other TLRs like TLR6 or TLR1 is required to activate TNF-α production in macrophages (202). Moreover, evidence exists that TLR2 is directly involved in bacterial killing by monocytes and macrophages (203).

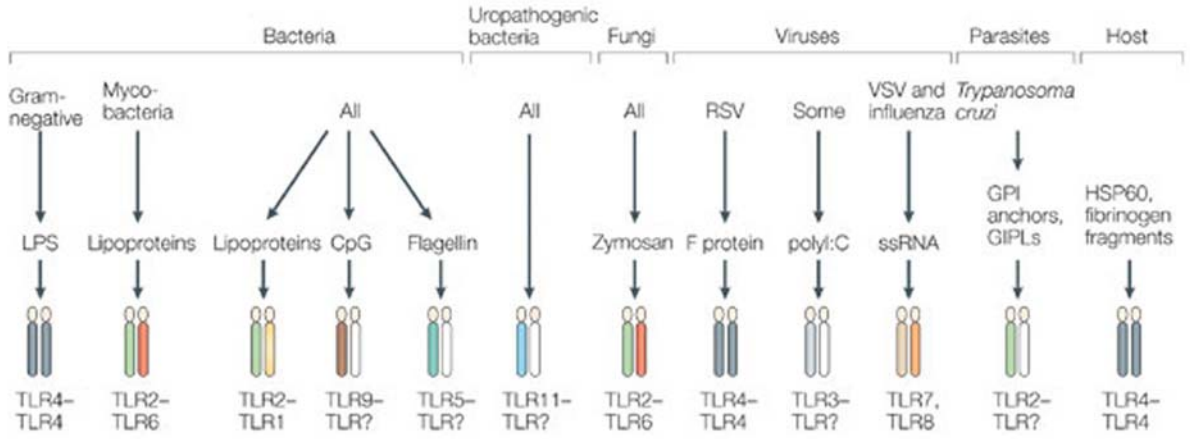

Figure 1.4. Toll like receptors and their ligands. Bacteria are sensed by five Toll-like receptors (TLRs) in humans: lipopolysaccharide (LPS) is the main bacterial ligand for TLR4; lipotechoic acid and diacylated lipopeptides are sensed by a TLR2–TLR6 dimer; triacylated lipopeptides are sensed by a TLR2–TLR1 dimer; CpG motifs are sensed by TLR9; and flagellin is sensed by TLR5. In mice, an additional TLR, TLR11, is also involved in anti-bacterial responses and senses uropathogenic bacteria. For anti-fungal responses, a TLR2–TLR6 dimer senses zymosan. Five TLRs are involved in anti-viral responses: TLR4 senses F protein from respiratory syncytial virus (RSV); double-stranded RNA (polyI:C) is sensed by TLR3; TLR9 senses viral CpG DNA (not shown); and TLR7 (human only) and TLR8 (human and mouse) sense single-stranded viral RNA (ssRNA). Protozoal products such as glycosylphosphatidylinositol (GPI)-anchor proteins are also sensed by TLR2. Finally, products of inflamed tissues such as heat-shock protein 60 (HSP60) are sensed by TLR4. This repertoire means that almost all pathogens that infect humans will be sensed by TLRs. GIPL, glycoinositolphospholipid; VSV, vesicular stomatitis virus (Illustration from Nature Reviews Immunology (198).

1.6.1 Signalling via TLRs

The activation of TLR signalling pathways originates from the cytoplasmic TIR domains. This involves a proline residue in the TIR domain that is conserved among all TLRs, except for TLR3, and its substitution to histidine caused a dominant negative effect on TLR-mediated signalling (201;204;205). In the signalling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, was first characterized to play a crucial role. In addition, recent accumulating evidence indicates that TLR signalling pathways consist, at least, of a MyD88-dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is peculiar to the TLR3 and TLR4 signalling pathways (206). Analysis of the MyD88-independent pathway led to the identification of two adaptors; TIRAP (TIR domain-containing adaptor protein) and TRIF (TIR domain-containing adaptor inducing IFN-β). These two adaptors regulate the TLR-mediated signalling pathways by providing specificity for individual TLR signalling cascades (207;208;197;209). Signal

transduction processes activated by Toll-like receptors (TLRs) include the important transcription factor NF-κB and 2 MAP kinases, p38 and Jun N-terminal kinase. These signals ultimately give rise to increased expression of a multitude of pro-inflammatory proteins (197;210). TLRs are expressed constitutively or inducibly on a variety of cells such as monocytes / macrophages, dendritic cells (DCs), B cells, T cells, mast cells, and endothelial cells but the pattern of expression of individual TLRs differs for specific cell types (211).

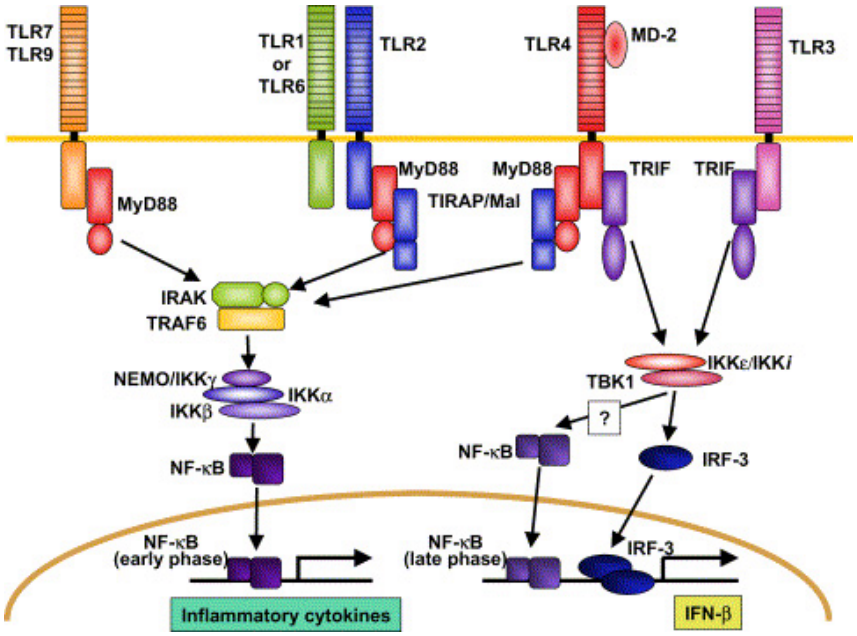

Figure 1.5. TLR-mediated pathways. MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual TIR domains. Upon stimulation, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor, which induces association of IRAK-1 and MyD88 via the death domains. IRAK-4 then phosphorylates IRAK-1. Phosphorylated IRAK-1, together with TRAF6, dissociates from the receptor and then TRAF6 interacts with downstream kinases such as TBK1 that phosphorylates the IKK complex, consisting of IKKα, IKKβ, and NEMO/IKKγ thereby induces the activation of the transcription factors NF-κB that leads to the induction of inflammatory cytokines. TIRAP is a second adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. In the TLR4- and TLR3-mediated signaling pathways, a MyD88-independent pathway exists mediated by TRIF that leads to activation of IRF-3 and IFNβ via TBK1 and IKKε/IKK*i*. (Illustration from (197).

1.6.2 Role of TLRs in signal induction by *L. monocytogenes*

There is ample evidence to suggest that *L. monocytogenes* interacts with several TLRs. As mentioned above, TLR2 recognizes Gram-positive bacterial compounds such as peptidoglycan, Lipotechoic acid and lipoproteins (198). That TLR2 is a signalling receptor for *L. monocytogenes* products was revealed based on in vitro studies carried out on Chinese hamster ovary cells transfected with TLR2 or cells from TLR2 deficient mice (212;213). So far, the strongest evidence for the significance of TLR signalling by *L. monocytogenes in vivo* comes from infection studies done on mice deficient in MyD88 the common adaptor protein of TLR signalling. MyD88$^{-/-}$ mice are highly susceptible to *Listeria* infection and demonstrate diminished capacity to secrete cytokines, NO release, as well the cell surface expression of costimulatory molecules in response to *Listeria* infection (213;214). Although there is a general consensus on the role of TLR2 in signal induction by *L. monocytogenes* products, its *in vivo* relevance is still controversial. Infection experiments by Edelson and Unanue found that although TLR signalling is required for NO and cytokine production by macrophages, resistance to *Listeria* infection between the TLR2$^{-/-}$ and wild-type mice was equivalent (213). This is however in contrast to the more recent study in which TLR2$^{-/-}$ mice were shown to be significantly susceptible to *Listeria* infection (214). The discrepancy between these two studies could be due to the differences in experimental conditions such as the route of infection as well as the *Listeria* strain used. Notwithstanding above reasons, there is a growing appreciation that *L. monocytogenes* does also trigger signalling via other TLRs, thus causing some redundancy in TLR recognition. In principle, TLRs 1, 2, 4, 5, 6 and 9 may be involved in the in vivo response to live Listeria through recognition of lipoprotein (heterodimer of TLRs 1 and 2) (198;199;215), peptidoglycan (heterodimer of TLR2 and TLR6) (202;216), lipotechoic acid (TLR4 (216), *Listeria* flagellin (TLR5) (198;217). Bacteria DNA (199;218).

The signals triggered by LLO could also contribute to redundancy in TLR signalling by *L. monocytogenes*. NF-κB is the key transcription factor through which TLR signalling activates proinflammatory genes. As shown below, NF-κB is also one of the major transcription factors activated by LLO thus indicating a convergence between the TLRs and LLO activated signalling pathways. A recent studies has reported that LLO and other cholesterol dependent cytolysins induce inflammatory response in host cells in a TLR4 dependent manner (219;220). How and at what

level the LLO and TLR signals converge are unresolved questions which could help to shade more light into this redundancy in the signals induced in the host by *L. monocytoges* and other Gram-postive bateria that express cholesterol dependent cytolysins.

1.7 Objectives of the work

The overall aim of this work is to elucidate the mechanisms of signal induction by Listeriolysin O, the major virulence factor of *L.monocytogenes,* in order to understand how such signals influence the course of listeriosis.

As outlined in the introduction, LLO triggers signals in host cells via pore and non-pore dependent mechanisms. One of the pore dependent mechanisms of signal transduction by LLO is Ca^{2+} flux induction. So far, Ca^{2+} signal induction by LLO has largely been deduced based on studies carried out using bacterial preparations (72;80). Since Ca^{2+} signals can be induced by other components such as PI-PLC*,* no conclusive studies have so far been carried out to specifically delineate the precise mechanisms of Ca^{2+} induction by LLO.

- The first objective of study was to dissect the mechanism of Ca^{2+} signalling by LLO. In particular, whether LLO also triggers Ca^{2+} release from intracellular stores in addition to Ca^{2+} influx, and the underlying mechanisms have been investigated. For that, recombinant LLO purified from the non-pathogenic *Listeria innocua* strain, which does not express PI-PLC - a potential contaminant that could complicate interpretation of Ca^{2+} signals induced by LLO, was used.
- The second objective was to delineate the pore independent mechanisms of signal induction by LLO. Since LLO is a cholesterol dependent cytolysin and cholesterol is the mains structural component of lipid rafts, whether LLO signals via lipid rafts and the underlying mechanisms have been investigated.
- The third objective was to investigate the functional outcome of signal induction in host cells by LLO, and how such signals shape the course of listeriosis. The studies in this part were largely focused on the cellular responses triggered by LLO in mast cells both *in vitro* as well as *in vivo*.
- In addition to LLO, several other bacterial components also contribute to the overall host responses triggered by *L. monocytogenes.* One of the prominent

signaling pathways activated by bacterial pathogens are those via Toll like receptors (TLRs). As such, TLR deficient animals were analysed with the aim of understanding whether and how the LLO induced signals synergizes with those activated via the TLRs to shape host`s response during *Listeria* infection.

Taken together, it was considered if carried out, these studies would yield important information that would further the understanding of how *L. monocytogenes* interacts with the host.

2 Materials and methods

2.1 Animals

Female BALB/c mice were purchased from Harlan (Borchen, Germany) and used at an age of 10-12 weeks. C57BL/6 mice and MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR2/4$^{-/-}$ mice were a gift from Dr. Uwe Klemm (Max Planck Institute for Infection Biology, Berlin, Germany).

2.2 Antibodies and reagents

Mouse anti-HA antibodies (HISS-Diagnostics, Freeburg, Germany), CT-B and polyclinic goat anti CT-B (Caliches, Darmstadt, Germany). Cy3-streptavidin and peroxidase-goat anti-rabbit (Jackson Immunoresearch Laboratories, Hamburg, Germany), FITC-rat monoclonals anti-mouse CD14 (mrC5-3), rat anti-mouse transferrin receptor (TFR), streptavidin-FITC (BD Pharmingen, Heidelberg, Germany). Goat anti-rat and goat-anti rabbit antibodies coupled to alexa488 were obtained from Molecular probes, mouse anti-phosphotyrosine (PY99), rabbit anti-Lyn and rabbit anti-Syk were obtained from Santa Cruz Biotechnology. Rabbit anti-phosphotyrosine (Chemicon International Temecula CA USA), anti-rabbit IgG-FITC (Sigma Immunochemicals, Deisenhofen, Germany), filipin (Sigma-Aldrich Chemicals, Steinheim, Germany) were obtained from the indicated venders. Cy2-donkey anti-goat Ig was a kind gift from Dr. D. Monner (GBF, Braunschweig, Germany). Wild type LLO and HA-LLO were purified from Wisteria innocuous hyper-expressing strains (221). p-nitro phenyl-N-acetyl-β-D-glucosaminide and tharpsigargin were obtained from Sigma; while Indo-1-AM, BAPTA-AM and ER-tracker, were from Molecular Probes. Dulbecco's modified Eagle's medium (DMEM) and Calcium free DMEM were from Gibco.

2.3 Cell lines and primary cells

J774 cells were from our cell line collection. Rat basophilic leukemia cell line (RBL-2H3) was kindly provided by Prof. Pecht I (The Weizmann Institute of Science, Israel). Bone marrow derived mast cells (BMMC) were generated as follows: Bone marrow cells were harvested by repeatedly flushing femurs and tibias with Iscove's

modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS; inactivated at 56°C), 2 mM L-glutamine, 1 mM pyruvate, 100 U of penicillin/ml, 100 µg of streptomycin/ml, 20 U of mIL-3/ml (IMDM). The cell culture was established at a density of 2 x10^6 cells/ml in IMDM. Nonadherent cells were transferred every 2 to 3 days in to fresh plates with 20% of their accustomed medium. After 4 weeks of culture, more than 99% of cells were identifiable as mast cells as determined by toluidine blue staining and flow cytometric analysis of cell surface expression of c-kit and FcεRI.

2.4 Depletion of neutrophils and mast cells in mice

Except for minor changes depletion of neutrophils or mast cells was done according the published protocols (222) and (223) respectively. Briefly, to deplete BALB/c mice of their mast cells, mice were given an intraperitoneal injection of 1.5 mg of anti-c-Kit monoclonal antibody (ACK2) the day before each challenge with *L. monocytogenes.* On the other hand, depletion of neutrophils in mice was accomplished by injecting them intraperitoneally with 100 µg of the RB6-8C5 MAb. Control mice were injected intraperitoneally with equivalent amounts of rat immunoglobulin G instead of the ACK2 or RB6-8C5 antibodies.

2.5 Ca^{2+} flux measurements by flow cytometry

5 x 10^6 BMMCs in 500 µl DMEM were incubated with 50 µM INDO 1-AM for 45 min at 37°. Cells were then washed three times in 5 ml Ca^{2+} free DMEM. In the last washing steps the cells were partitioned into two, and the aliquots washed and resuspended in either normal or Ca^{2+} free medium. Cells kept on ice until ready for calcium measurement were warmed up to 37°C and the cytosolic Ca^{2+} was determined for the first 30 sec and then continued after addition of the stimulus. For the FcεRI cross-linking, INDO 1-AM labelled cells were incubated with an anti-BSA-DNP Age (5 µg/ml) for 30 min on ice then washed. The FcεRI was cross-linked by adding BSA-DNP (10 µg/ml). Measurements were carried out on a MoFlo high-speed cell sorter (DakoCytomation USA) equipped with an UV argon ion laser (351-363 nm). INDO-1 emissions were detected with 405/30 (Ca-bound INDO-1) and 515/30 (Ca-free INDO-1) fluorescence filters, and the ratio of bound / free was recorded.

Data were analysed by FloJo software (Tree Star, San Carlos, USA). Data were normalized for the fluctuations in the starting measurements by the Excel software.

2.6 Measurement of β-Hexosaminidase activity

Release of β-hexosaminidase was measured as an index of mast cell degranulation using a standard method (224). In brief, 2 x 10^5 BMMCs in 200 µl IMDM were seeded into each well of a 96 well plate then stimulated with either LLO/CL-LLO (0.25 µg/ml) or infected with *L. monocytogenes* (MOI 100). After incubation at 37°C for 2 hours, the supernatants were transferred to a 96-well plate. The rest of the supernatants were removed, and cells were lysed in 0.5% Triton X-100 solution. To determine the β-hexosaminidase content of supernatants and cells 50 µl of samples were incubated with 50 µl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) dissolved in 0.1 mM citrate buffer, pH 5.0, in a 96-well microtitre plate for 1h at 37°C. The reaction was stopped by adding 150 µl of 0.1 M carbonate buffer, pH 10.5. Optical density was measured at 405 nm in a microplate reader (BIO-RAD), and the net percentage of the β-hexosaminidase released was calculated as follows: β-hexosaminidase in supernatant/ (β-hexosaminidase in supernatant + β-hexosaminidase in cells) x 100.

2.7 Infection of mast cells with *L. monocytogenes*

After incubating with *L. monocytogenes* at a multiplicity of infection (MOI) of 100 for 1 hour, the BMMCs were washed then incubated for additional 3-4 hours in the presence of 10 µg/ml gentamycin. Thereafter, the cell pellet and the culture supernatants were collected for RT-PCR analysis and TNF bioassay, respectively.

2.8 TNF Bioassay

Following stimulation of BMMC with LLO or infection with *L. monocytogenes*, the biological activity of TNF in the supernatants was assayed using the TNF-sensitive murine fibroblasts L929. Briefly: 4x10^4 cells were seeded into each well of a 96-well plate. After overnight incubation, the medium was replaced with 100 µl of medium containing actinomycin D (6.25 µg/ml) and 50 µl of the sample supernatant was added. Serial dilutions of recombinant TNF-α were also used as reference. After

incubating the plate for 24 h the supernatants were aspirated and cell viability determined using the EZ4U KIT (BIOMEDICA, Viena, Austria) according to the manufacturer's instructions. The TNF concentration was determined by comparing the L929 killing induced by the samples with killing induced by dilutions of TNF-α of know concentrations.

2.9 RT- PCR analysis

mRNA was isolated from BMMCs using the RNAEasy Mini Kit from Qiagen and reverse transcribed into cDNA using the SuperScript II RNAseH Reverse Transcriptase kit from Invitrogen. PCRs were performed with primers: (5`-3`) TNF-α: (TCT CAT CAG TTC TAT GGC CC; GGG AGT AGA CAA GGT ACA), MCP-1: (GCCCCACTC ACCTG CTGCTA; TTTACGGGTCAACTTCACATTCAA), MIP-1α: (CTGCCCTTGCTGTTCTTCTCTGTA; GATCTGCCGGTTTCTCTTAGTCA) and the house keeping gene RPS9: (CTG GAC GAG GGC AAG ATG AAG C; TGA CGT TGG CGG ATG AGC ACA). Amplification conditions were: denaturation at 94°C for 1min followed by 27 and 32 cycles (for RPS9 and TNF-α respectively of repeated denaturation (20s at 94°C), annealing (20s at 58°C) and extension (20s at 72°C). PCR products were analyzed on a 2 % agarose gel.

2.10 Neutralization of LLO with antibody and cholesterol

To make the LLO+M344 complex, 10 µg of LLO was incubated with 0.5 mg of the M344 antibody in a 100 µl PBS (i.e. 0.1 mg/ml LLO in 5 mg/ml of antibody solution) for 45 min at room temperature. To make CL-LLO+M344, 5 µl of cholesterol stock (4 mg/ml in chloroform) was added to 95 µl of the LLO+M344 solution then vortexed thoroughly.

To treat cells, 5ul of either the LLO+M344 or CL-LLO+M344 solutions was added to cells on cover slips immersed in 500 µl of tissue culture medium. This brings the final concentration of LLO to 1 µg/ml while that of cholesterol to 2 µg/ml. After 10 min incubation the cells were washed, fixed then stained. Cells treated with this concentration of cholesterol alone showed no visible difference with the untreated.

2.11 Preparation of Detergent Resistant Membranes (DRMs)

Monolayers of J774 cells (about 10^7 cells /200 ml tissue culture flask) were incubated with 2 µg/ml LLO or CL-LLO for 15 min at room temperature (RT). Subsequently cells were scrapped off the flasks, washed in ice cold PBS, then lysed at 4°C in 1 ml 1 % Triton X-100 buffer (5 mM EDTA, 25 mM Tris pH 7.5, 0.15 M NaCl, 1% Triton X-100 plus a cocktail of protease inhibitors (Roche Diagnostics Mannheim, Germany). Lysates were adjusted to 40% sucrose by adding 1 ml of 80% sucrose in 1% Triton X-100 buffer before over layering with 11 ml of 35% sucrose, then 4 ml of 16% sucrose. After centrifugation at 150,000 x g for 24 h at 4°C in a SW 28.1 rotor (Beckman Instruments, München, Germany), 12 equal fractions collected from top to bottom were precipitated in 10% Trichloroacetic acid (TCA). After estimating the protein concentration in each fraction, equal amounts were loaded on the gels and analysed by immunoblotting for LLO, CD14, CD16, CD24 and TFR.

2.12 Separation of monomeric and oligomeric LLO

Separation of monomeric and polymeric LLO from J774 cells was carried out as previously described (66;225). Briefly: $5x10^6$ cells in 100 µl PBS were incubated with LLO at a concentration of 10 µg/ml for 15 min at RT. After lysis in 1 ml of 250 mM DOC (250 mM deoxycholate, 20 mM Tris HCl, 1 mM EDTA pH 8.0), the cell lysates were applied to a continuous 10-50% sucrose gradient (17 ml) containing 6.25 mM deoxycholate, 20 mM Tris-HCl, 1 mM EDTA pH 8.0 then centrifuged for 3 h at 150 000 x g using a SW 28.1 rotor at 4 °C. 11 equal fractions were collected from top and precipitated in 10% TCA then analysed for LLO by immunoblotting. Purified LLO and CL-LLO were fractionated and analysed in parallel.

2.13 Immunofluorescence staining and microscopy of J774 cells

Two procedures were used for fixation; incubation in 4% paraformaldehyde for 15 min at room temperature (PFA fixation) or PFA fixation followed by 10 min incubation in a 1:1 acetone/methanol mixture at -20°C (PFA/Ac-MeOH fixation).

J774 cells grown on cover slips were incubated with HA-LLO (1 μg/ml in incomplete IMDM) for 10 min at room temperature (RT), washed, then fixed. Alternatively, cells were first fixed before incubation with HA-LLO. Cell surface GM1, was labeled with 10 nM CT-B for 40 min at RT after fixation.

Before incubation with antibodies, the fixed cells were blocked in 0.5% BSA/PBS for 30 min (RT). HA-LLO was revealed using a mouse anti-HA (1:2000) or a biotin-coupled anti-HA (1:4,000) followed by a goat anti mouse-Cy3 (1:2,000) or Cy3-streptavidin (1:5000) respectively. CT-B was stained using a polyclonal goat anti-CT-B followed by Cy2-donkey anti-goat Ig. For double staining of HA-LLO and CD14 (1:50), cells treated with HA-LLO or CL-HA-LLO were fixed in PFA or PFA/Ac-MeOH, before incubation for 1 h on ice with either (1) mouse anti-HA-biotin antibody followed by Cy3-streptavidin and FITC-rat anti-CD14 or (2) a mixture of mouse anti-HA (1:2,000) and rat anti-CD14 followed by a mixture of Cy3-goat anti-rat(1:2,000) and alexa488 coupled goat anti-rat (1:100). The TRF was stained using a rat anti-TFR followed by alexa488 coupled anti-rat (1:100). HA-LLO and CD16 on PFA/Ac-MeOH fixed cells was revealed using anti-HA and rat anti-CD16 followed by Cy3- anti-mouse and alexa488-anti-rat.

For the co-clustering of HA-LLO and Lyn, J774 cells treated with 1 μg/ml HA-LLO were fixed by PFA/Ac-MeOH before staining with anti-HA and rabbit anti-Lyn (1:100) followed by Cy3-anti-mouse and alexa488 coupled goat anti-rabbit antibodies. For the HA-LLO and tyrosine phosphorylation experiments, J774 cells were cultured for 24 hrs in incomplete medium before incubation with HA-LLO (0.25 μg/ml) for 5 min at 37°C. After fixation, cells were stained for HA-LLO as described above then permeabilized using 0.1% Triton X-100 /PBS for 1 min. Subsequently, cells were blocked with 3% BSA/PBS for 30 min (RT), then stained for phosphotyrosines using a rabbit anti-phosphotyrosine antibody followed by a FITC-goat anti rabbit Ig.

Cover slips were mounted onto slides using Flouprep mounting medium (bioMérieux, Marcy l´Etoile, France) then examined using an Axiovert 135 TV microscope (Zeiss, Oberkochen, Germany) equipped with Cy3, FITC filters and a Plan-Apochromat 100X/1.40 NA oil immersion objective. Images were recorded with a cooled (-25°C), back-illuminated CCD camera.

Images were processed using VayTek Microtome digital deconvolution (Fairfiel IA USA) and Adope Photoshop softwares (Adope Systems, Inc., Mountain View, CA USA) on a Macintosh computer (Apple Computer Co., Cupertino CA USA).

For quantitative colocalization, fluorescent images were captured by scanning laser confocal microscopy using a Plan Apo lens (40 x, NA 1.4, oil immersion) and analysed with Zeiss LSM 510 software (Carl Zeiss, Jena, Germany).

2.14 Mast cells staining and microscopy

BMMCs untreated or treated with LLO (0.25 µg/ml) or ionomycin (1 µM) for 45 min, were pelleted, then fixed and permeabilized by incubation in a 50:50 Acetone/methanol fixative for 10 min at –20°C. Cells were blocked in 3% BSA for 30 min then stained with a mouse anti-NFATc1 (Santa Cruz Biotechnol. CA) for 45 min at RT. After washing, the cells were incubated with a Cy3-Goat anti-mouse antibody for 45 min. Cells were again washed, transferred onto slides by cytospining and cover slips were mounted onto them using Fluoprep mounting medium.
For the time series movies, RBL-2H3 mast cells grown on cover chamber slides were labelled with 2 µM BODIPY ER-tracker in phenol red free medium for 30 min at 37°C. After washing, fluorescent images of the cells were captured at 37°C at an interval of 30 sec by scanning laser confocal microscopy using a Plan Apo lens (40x, NA 1.4, oil immersion). The time series of the images was converted into an avi movie using the Zeiss LSM 510 software (Carl Zeiss, Jena, Germany).

2.15 Immunoprecipitation and western blot analysis

J774 cells grown for 24 hrs in incomplete IMDM medium to confluency (10^7 cells/ 200 ml culture flask) were stimulated with 0.25 µg/ml HA-LLO at 37°C. After 2 and 4 min, cells were scrapped off, washed in ice cold PBS and lysed in 600 µl 1% Triton-X 100 extraction buffer (described above) supplemented with 2 mM Na_3VO_4, 10 mM NaF and a cocktail of protease inhibitors. After 15 min on ice, the soluble and insoluble materials were separated by centrifugation at 20,000g for 30 min. The pellet containing the insoluble materials (designated as DRM) was washed 3x with 1% Triton-X 100 extraction buffer and then solubilized on ice for 30 min in 600 µl of RIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 2 mM Na_3VO_4 and 10 mM NaF containing a cocktail of protease inhibitors).
For immunoprecipitation 500 µl of each of the fractions was filled up to 1 ml and then incubated at 4°C with 1µg of the anti-phsophotyrosine antibody (PY99). After 2 hours 20 µl of ProteinA/G-Agarose was added and the lysates incubated overnight at 4°C.

The Agarose pellet were washed 3 times in RIPA buffer then boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblotting using rabbit anti-Lyn and rabbit anti-Syk as developing reagents. Sample aliquots of pre-immunoprecipitation lysates were also analyzed for phosphotyrosines, Lyn and Syk.

3 Results

Signal induction in host cells is an essential prerequisite for the survival of pathogens in the host. Thus many of the virulence factors of pathogens are devoted to such activity. Listeriolysisn O (LLO) is a paradigm of such virulence factors that is not only essential for the escape of the bacterium into the cytosol but more importantly induces many cellular responses in the target cells of *L. monocytogenes.* The goal of the present study was to shed some light into the mechanisms that underlie signal induction by LLO. The results presented below are in three parts. The first part (section 3.1) deals with the mechanisms of calcium induction by LLO while the second part (section 3.2) covers the role of lipid rafts in the induction of signalling by LLO. The third part (section 3.3) deals with the functional outcome of signal induction by LLO in mast cells and their significance *in vivo* during *Listeria* infection. The fourth part (section 3.4), deals with how the signals triggered by LLO synergize/overlap with those triggered by other listerial components that engage TLRs and how this determines the overall host response to *Listeria* organism.

3.1 Induction of calcium signals in host cells by LLO

As discussed above, induction of calcium signals in host cells has emerged to be a common feature employed by various bacterial pathogens to communicate with their target host cells. Previous studies have shown that *L. monocytogenes* induce calcium signals in host cells in an LLO dependent manner (71;80;88). The present studies were carried out with the aim of delineating the mechanism underlying calcium signal inducton by LLO.

3.1.1 LLO induces release of Ca^{2+} from intracellular stores in mast cells

Mast cells have been studied mainly in relation to allergic diseases. However, the importance of these cells in the control of pathogens is now well recognised (226). Mast cells are endowed with at least two important properties which are instrumental in the host defence: (i) their strategic location at the host-environment interfaces which are the ports of entry for pathogens; (ii) in response to the appropriate stimuli, mast cells rapidly secrete preformed pro-inflammatory mediators stored in granules.

They are also able to quickly synthesize such mediators upon stimulation. Since degranulation and synthesis of pro-inflammatory mediators by mast cells can be regulated by Ca^{2+} signals, mast cells were considered an ideal cell type to conduct functional and mechanistic studies on the induction of Ca^{2+} fluxes by LLO.

To investigate the mechanism of Ca^{2+} signals by LLO, bone marrow derived mast cells (BMMCs) were stimulated with the toxin in normal or Ca^{2+} free medium and Ca^{2+} flux was analysed by flow cytometry. LLO was found to elevate cytosolic Ca^{2+} in mast cells both in the presence and absence of extracellular Ca^{2+} (Figure 3.1A). Ca^{2+} elevation in Ca^{2+} free medium was lower indicating that in normal medium, the magnitude of Ca^{2+} elevation by LLO is a product of influx of extracellular Ca^{2+} and release from intracellular Ca^{2+} stores. The calcium ionophore ionomycin also triggered Ca^{2+} elevation in normal as well as in Ca^{2+} free medium. Compared to LLO however, Ca^{2+} induction by ionomycin was almost immediate (Figure 3.1A). The delayed kinetic of Ca^{2+} induction by LLO probably illustrates the critical time frame required for the transformation of LLO monomers into functional pores.

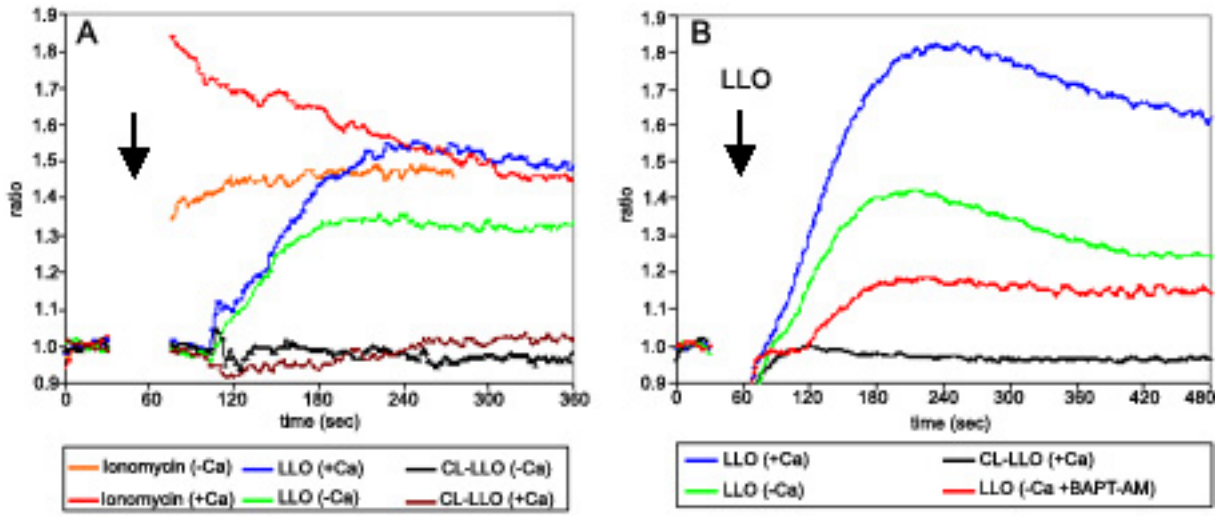

Figure 3.1. LLO induces influx of Ca^{2+} as well as release from intracellular stores. (A) Indo 1-AM loaded BMMCs were stimulated with LLO or ionomycin in normal or Ca^{2+}-free medium. **(B)** BMMCs were incubated with BAPT-AM for 37°C for 30 min to chelate intracellular Ca^{2+}. After washing, cells were loaded with Indo 1-AM before stimulation with LLO in normal or Ca^{2+}-free medium. A 30 sec baseline was recorded each time before stimulation. The arrows indicate the time points of stimulation.

Preincubation of LLO with cholesterol abrogates its ability to form pores but not its membrane binding (66;112;185). When tested, cholesterol inactivated LLO (CL-LLO) was found not to induce any Ca^{2+} flux in the BMMCs (Figure 3.1A). This suggests

that in such cells, the influx and release of Ca^{2+} from intracellular stores occurs exclusively via pore dependent mechanisms. To confirm the release of Ca^{2+} from intracellular stores, cells were also stimulated with LLO after the chelation of intracellular Ca^{2+} using BAPTA-AM. A diminished Ca^{2+} response was obtained in BAPTA-AM pretreated cells, again emphasizing the contribution of the intracellularly stored Ca^{2+} to the overall Ca^{2+} response triggered by LLO (Figure 3.1B).

3.1.2 LLO and *L. monocytogenes* induce *de novo* synthesis and secretion of proinflammatory factors by mast cells

To evaluate the functional outcome of LLO induced Ca^{2+} fluxes, BMMCs were analysed for degranulation by measuring the secretion of hexosaminidase, an enzyme that is stored in the granules of mast cells and released upon degranulation. Both LLO and *L. monocytogenes* were found to induce release of hexosaminidase (Figure 3.2A). Consistent with the data on Ca^{2+} induction (Figure 3.1A), mast cell degranulation by LLO and *L. monocytogenes* was found to be lower in Ca^{2+} free medium while no degranulation was induced by CL-LLO (Figure 3.2A).

Tumor necrosis factor-alpha (TNF-α) is one of the preformed cytokines rapidly secreted by the mast cells via degranulation (226). Independent of that, mast cells also synthesize TNF-α in response to the appropriate stimuli. Therefore, whether LLO and *L. monocytogenes* activate TNF-α gene transcription was also tested. Analysis by RT-PCR demonstrated that both LLO and *L. monocytogenes* upregulate TNF-α mRNA with a peak response at 3-4 h post stimulation (Figure 3.2B). The higher level of specific mRNA elicited by bacteria indicates that, in addition to LLO, other virulence factors also contribute to this response. The accumulating TNF activity in culture supernatants of the corresponding samples revealed that the specific TNF-α mRNA is indeed translated and TNF-α is secreted following stimulation by LLO and *L. monocytogenes* (Figure 3.2C).

3.1.3 The TNF-α gene transcriptional activation by LLO is pore dependent, occurs via NFAT activation, and occurs independently of extracellular Ca^{2+}

What is the significance of Ca^{2+} signalling on the TNF-α gene transcriptional activation by LLO? When BMMCs were stimulated in normal or Ca^{2+} free medium, LLO was found to induce upregulation of TNF-α mRNA in both cases while CL-LLO, which does not induce Ca^{2+} signals, elicited either a minor or no response (Figure 3.3A). This suggests that induction of Ca^{2+} signals by LLO plays a major role in the activation of the TNF-α gene. The Nuclear Factor of Activated T cells (NFAT), a calcium dependent family of transcription factors, is involved in the regulation of the transcription of a broad range of cytokines and chemokines.

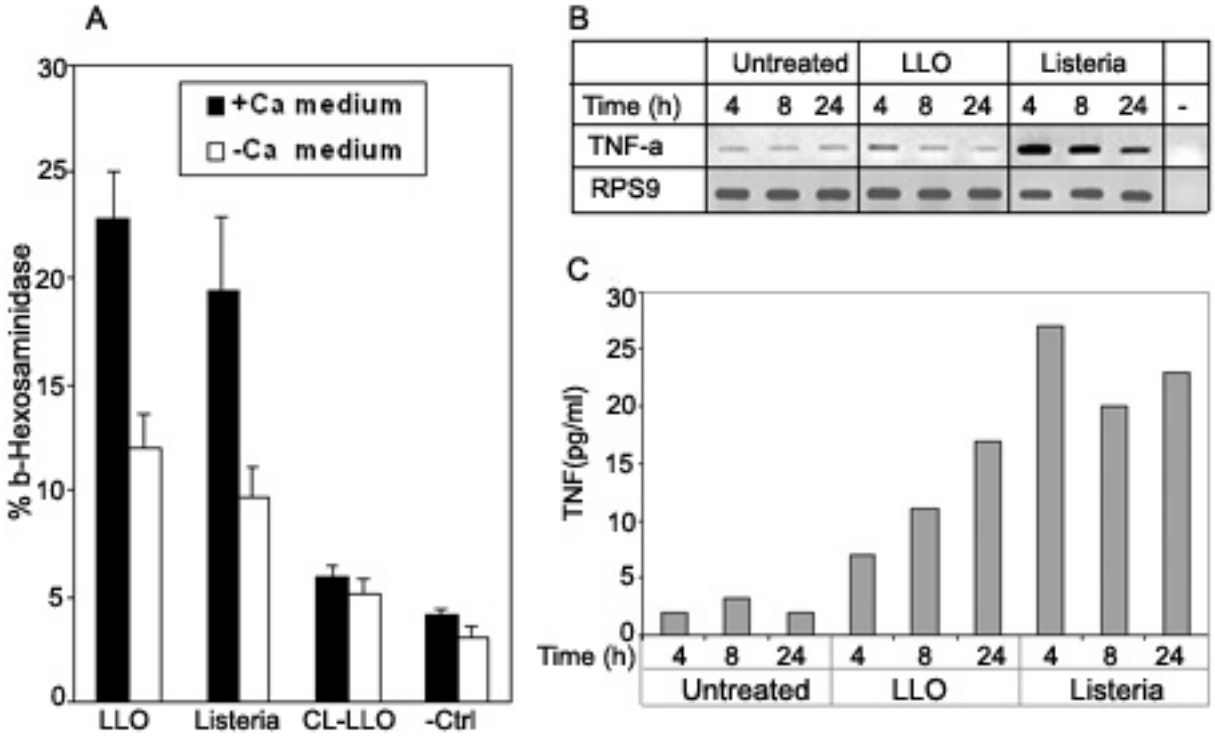

Figure 3.2. LLO and *L. monocytogenes* induce *de novo* synthesis and secretion of pro-inflammatory factors in mast cells (A) Degranulation was analysed in untreated BMMCs or following incubation with LLO or cholesterol inactivated LLO (CL-LLO) or *L monocytogenes*. β-hexosaminidase activity in the culture supernatants was measured as an indictor of degranulation. BMMCs were either treated with LLO, or infected with *L. monocytogenes*, and at the indicated time points, the cell pellets were collected and analyzed by RT-PCR for TNF-α mRNA **(B)**. The mRNA of the house-keeping gene RPS9 was used as an internal control for the amount of cDNA used. For the secreted TNF-α, culture supernatants of the cells in **(B)**, were collected and tested in a bioassay **(C)**.

To determine whether LLO activates transcription of the TNF-α gene via NFAT, first, whether or not LLO activates the translocation of NFAT to the nucleus, was tested.

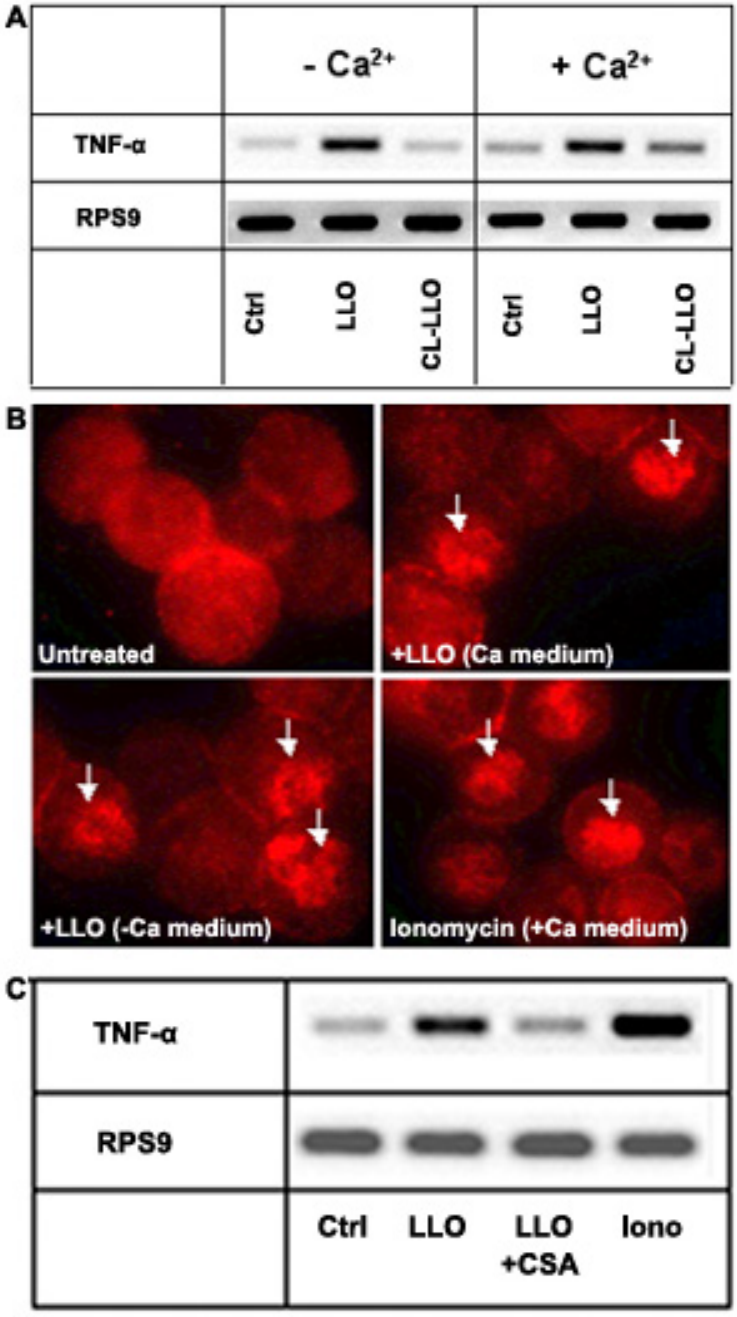

Figure 3.3. The TNF-α gene transcriptional activation by LLO is pore dependent, occurs via NFAT activation, and occurs independently of extracellular Ca^{2+}. (**A**) BMMCs were analyzed for TNF-α mRNA upregulation by RT-PCR following a 4 h stimulation with LLO or in normal or Ca^{2+} free medium. (**B**) BMMCs were left untreated or stimulated with ionomycin or LLO for 45 min at 37°C in normal or Ca^{2+} free medium. Permeabilized cells were then stained with an anti-NFATc1 antibody. The arrowheads indicate NFATc1 accumulated in the nuclei. (**C**) BMMCs were left untreated (Ctrl) or stimulated with LLO or ionomycin for 4 h in Ca^{2+} free medium in the presence/absence of 1 μM Cyclosporine A (CSA). Samples were analyzed for TNF-α mRNA upregulation by RT-PCR.

NFATc1 nuclear translocation was induced by LLO in normal as well as Ca^{2+} free medium (Figure 3.3B), indicating that Ca^{2+} released from the intracellular stores by LLO alone is sufficient to activate nuclear translocation of the NFAT family of transcription factors.

To explicitly demonstrate the role of NFAT in the induction of TNF-α gene transcription by LLO, BMMCs were stimulated with LLO in the presence of cyclosporine A (CSA), a potent inhibitor of NFAT activation. As shown in Figure 3.3C, blocking NFAT activation using CSA inhibited the upregulation of TNF-α mRNA by LLO. Taken together, these results show that: (i) LLO activates TNF-α expression via the calcium dependent family of transcription factors NFAT; and (ii) the Ca^{2+} released from intracellular stores by LLO alone is sufficient to trigger this response.

3.1.4 LLO penetrates the ER to cause release of ER components

Thus far, the data presented show that irrespective of Ca^{2+} influx, Ca^{2+}release from intracellular stores by LLO is sufficient to trigger cellular responses and that the lack of Ca^{2+} signals by CL-LLO suggests that the release from intracellular stores occurs via a pore dependent mechanisms. What then is the mechanism of Ca^{2+} release from the intracellular stores? As pointed out earlier, the large pores formed by CDCs allow the delivery of macromolecules as large as 400 kDa molecular mass into the cytosol without necessarily killing the cells (227;228). Given its pore dependent nature, whether Ca^{2+} release from intracellular stores was due the to secondary pores formed was considered in membranes of intracellular organelles subsequent to entry of LLO into the cytosol via the primary LLO pores in the plasma membrane, was considered.

The endoplasmic reticulum is the principal intracellular Ca^{2+} store. To test the above hypothesis, an experiment was designed that would allow to visually monitor whether LLO affects the ER directly. The ER-tracker BODIPY that accumulates in the ER lumen is a common tool for visualizing the ER in live cells. Because BODIPY leaks out of the ER in case of damage to the ER membrane, its retention within the ER lumen provides a means to evaluate the integrity of the ER. Therefore, to determine whether LLO perforates the ER, RBL-2H3 mast cells were loaded with ER-Tracker and then monitored for several minutes in the presence or absence of LLO. Upon treatment of cells with LLO, the ER was found to undergo rapid swelling accompanied by release of the ER-tracker (Figure 3.4A-B). This is in contrast to the

ER labelling of unstimulated cells, which was found to be steady throughout the time of image acquisition (Figure 3.4C-D). The Ca^{2+} ionophore ionomycin, which also induces Ca^{2+} influx as well as release from the intracellular stores, was however not found to release ER tracker from the ER (data not shown). Thus the LLO induced efflux of molecules from the ER was specifically due to the perforation of the ER by LLO.

It is noteworthy to mention that, despite these remarkable effects, when fresh medium was added, most of the cells (almost 80%) recovered from the effects of LLO and could proliferate for several days thereafter.

Taken together, these results suggest that LLO most likely penetrates the plasma and as well as the ER membranes to cause the release of ER components hence highlighting one of the mechanisms by which LLO, could release Ca^{2+} from intracellular stores.

3.1.5 Prolonged LLO treatment leads to the depletion of intracellular Ca^{2+} stores

Due to membrane repair mechanisms, cells treated with sublytic doses of toxin can fully recover. However, when cells were analysed for Ca^{2+} flux soon after treatment with LLO, surprisingly, it was observed that the LLO pretreated cells were refractory to calcium flux induction in response to LLO even in Ca^{2+} containing medium (Figure 3.5A).

A possible explanation for this phenomenon could be resistance of cells to membrane permeabilization. Such an effect was previously reported to be induced by pore forming agents (229). However, since pretreated cells were not resistant to propidium iodide uptake when re-treated with LLO (data not shown), resistance to permeabilization was ruled out as the cause of the diminished Ca^{2+} response. As shown earlier, the magnitude of Ca^{2+} response to LLO is a product of extracellular Ca^{2+} influx and release from intracellular stores. Having eliminated a decrease in Ca^{2+} influx, whether the above phenomenon was due to depleted intracellular Ca^{2+} stores was considered.

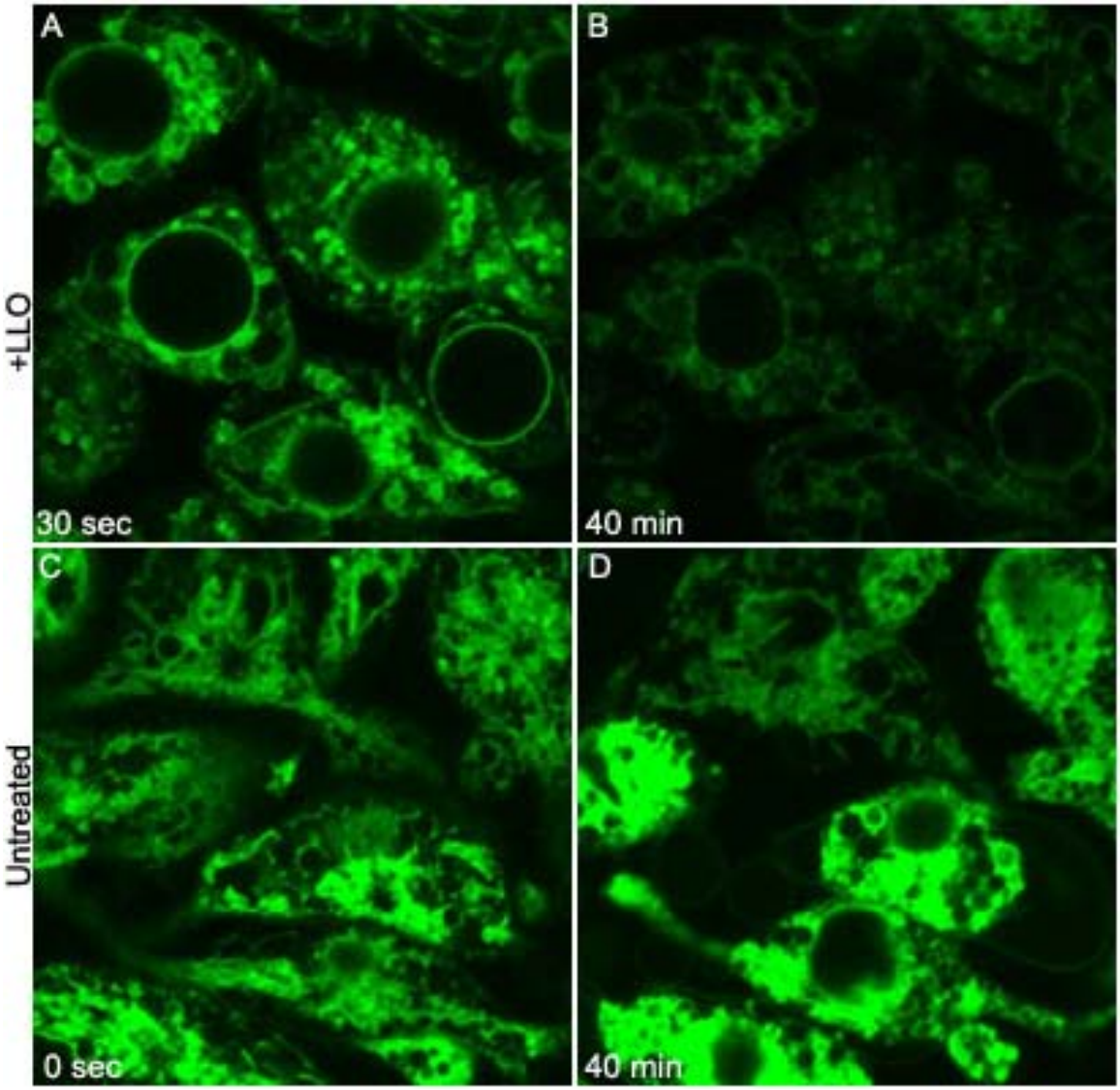

Figure 3.4. Perforation of the ER membrane by LLO causes the efflux of molecules from the ER lumen. To monitor release of molecules from the ER, the RBL-2H3 mast cells adhered on slides were loaded with the fluorescent ER-tracker. After washing, LLO was added to the cells and the efflux of ER tracker monitored for 40 min under a confocal microscope. The image in **A** and **B** shows the ER labeling in cells 30 sec and 40 min after addition of LLO respectively. **C** and **D**, respectively, show the labeling in untreated cells at the start (0 sec) and the end of a 40 min image acquisition.

When the experiments described above were repeated in Ca^{2+} free medium, remarkably low calcium levels were observed in the LLO pretreated cells (Figure 3.5B), an indication of depleted intracellular stores.

Cross-linking of the high affinity receptor for IgE, FcεRI, on mast cells results in the activation of tyrosine kinases with the subsequent generation of inositol triphosphate (IP_3), to trigger release of Ca^{2+} from internal stores. Therefore, to independently test the calcium depletion from the intracellular stores by LLO, calcium flux in response to

FcεRI cross-linking was tested. Again, as shown in Figure 3.5C, calcium release in response to FcεRI cross-linking was lower in LLO pretreated cells. In agreement, FcεRI cross-linking elicited almost no calcium response in cells pretreated with thapsigargin, an agent that also empties calcium from the intracellular stores.

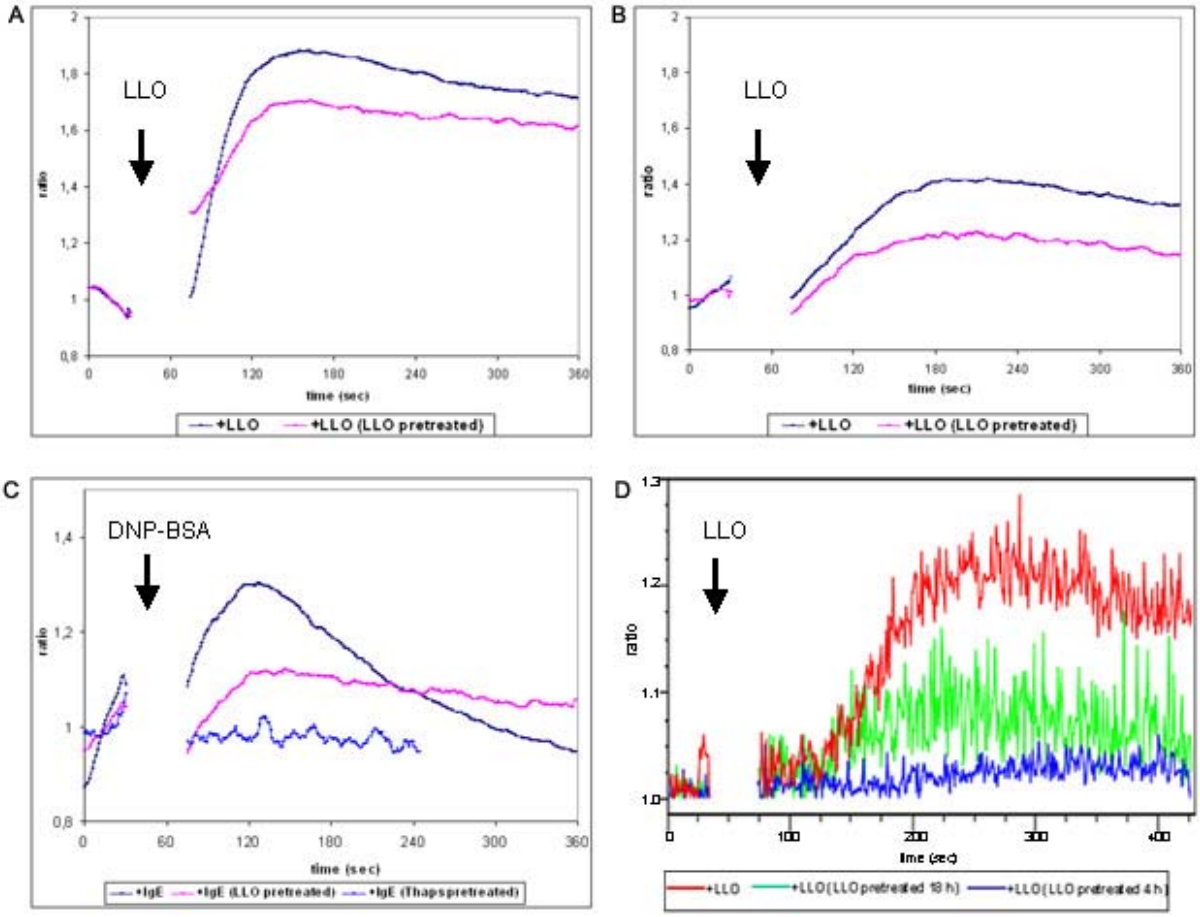

Figure 3.5. Pretreatment of cells with LLO for several hours depletes the intracellular Ca^{2+} stores thus rendering them refractory to subsequent stimulations. (**A-C**) After pretreating BMMCs with LLO or not for 4 h, cells were washed and loaded with Indo 1-AM. The elevation of cytosolic Ca^{2+} was evaluated in Ca^{2+} containing (**A**) and Ca^{2+}free medium (**B**). (C) BMMCs were left untreated or pretreated with LLO or thapsigargin (a reagent that depletes intracellular Ca^{2+} stores) for 4 h, loaded with Indo 1-AM then incubated on ice with with an IgE antibody specific for BSA-DNP. Cross-linking of the FcεRI was achieved by the addition of BSA-DNP. (**D**) Primary T cells pretreated with LLO for 4 h or 18 h to deplete the intracellular Ca^{2+} stores are refractory to Ca^{2+} induction by LLO. Cells pretreated (or untreated) with LLO for 4h or 18h were labeled with Indo-1-AM. Cytosolic Ca^{2+} elevation in such cells was then evaluated following their stimulation with LLO in Ca^{2+} free medium.

To demonstrate that depletion of intracellular calcium stores by LLO is a property that can be generalized to other cell types, primary T cells were also tested. In Figure 3.5D, untreated as well as T cells pretreated with LLO were re-stimulated with LLO in

Ca^{2+} free medium to assess only the Ca^{2+} released from intracellular stores. As shown before, calcium release from LLO-pretreated cells was remarkably lower than from untreated cells. Interestingly, almost no measurable calcium release was obtained with cells that were treated with LLO 4 h before measurement while cells treated 18 h earlier showed a low but definitive Ca^{2+} signal. This suggests that calcium depletion by LLO is reversible and that with time cells restock their intracellular Ca^{2+} stores.

Taken together, LLO induces Ca^{2+} signalling via the influx from the extracellular milieu and release from intracellular stores. The uncontrolled release of Ca^{2+} from the intracellular stores leads their depletion hence rendering them refractory to subsequent stimulation. This could have important physiological consequences in the host`s response to *Listeria* infection.

3.2 Interaction of LLO with Lipid Rafts

3.2.1 The role of cholesterol in the binding of LLO to plasma membranes

Previous studies showed the requirement of cholesterol in the interaction of LLO with artificial membranes (66). As discussed in the introduction, cholesterol is the main stractural component of lipid rafts. Thus, to evaluate whether LLO interacts with rafts, flow cytometry was used to study the effect of filipin on the binding of a HA-tagged LLO (HA-LLO) to J774 macrophages. Filipin binds to and sequesters membrane cholesterol from other interactions, thus, disrupting cholesterol dependent membrane microdomains (95). As shown in Figure 3.6A, filipin pre-treated cells display a diminished capacity to bind HA-LLO. In further experiments, J774 cells treated with β-methyl cyclodextrin (β-MCD), were tested for their capacity to bind HA-LLO. Unlike filipin, which only sequesters membrane cholesterol, β-MCD extracts cholesterol from plasma membranes.

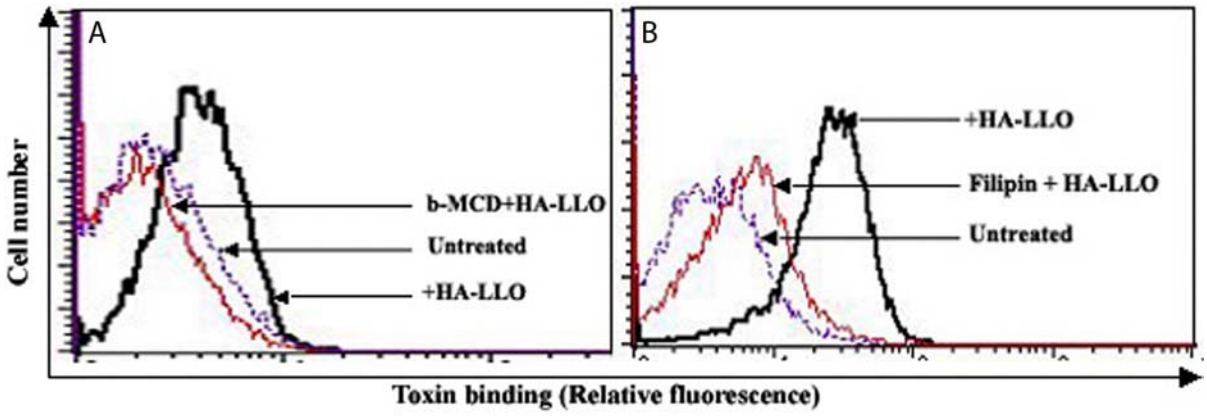

Figure 3.6. Depletion or sequestration of membrane cholesterol compromises HA-LLO binding to cells. J774 cells were treated with β-MCD or filipin (100 µg/ml in both cases) for 1h at 37°C before incubation with HA-LLO on ice. Cells were stained with biotinylated anti-HA antibody followed by streptavidin-PE then analysed by flow cytometry. The HA binding in represented as relative fluorescence intensity.

As illustrated in Figure 3.6A, treatment of J774 cells with β-MCD resulted in a complete abrogation of HA-LLO binding.

3.2.2 LLO partitions into Detergent Resistant Membranes (DRMs)

Cholesterol-rich microdomains or rafts are well recognised for their detergents insolubility - a property attributed in part to their cholesterol composition (111;230;231). Thus, if associated with rafts, cell bound LLO should partition into the detergent insoluble membrane fractions. J774 cells incubated with LLO were extracted with 1% Triton X-100 (on ice) and then subjected to ultracentrifugation on sucrose density gradients. Analysis of fractions by immunoblotting revealed that LLO preferentially partitioned into the DRMs that floated on the sucrose gradient (Figure 3.7A).

As discussed in the introduction, pre-incubation of LLO with cholesterol abrogates its cytolytic activity without compromising membrane binding (66). Therefore whether inactivated LLO also associates with the cholesterol-rich DRMs was investigated. Although CL-LLO exhibited a slightly different sedimentation pattern, the bulk of this toxin was also retained in the insoluble membrane fractions (Figure 3.7B). Further analysis revealed that the floating fractions were enriched in CD14 a GPI-anchored protein known for raft association (Figure 3.7A and B). In contrast, such fractions were depleted of the transferrin receptor (TFR), a membrane protein that is excluded from rafts (Figure 3.7A and B).

To address whether the detergent insolubility and buoyancy exhibited by LLO/CL-LLO is due to rafts association and not due to its intrinsic properties, LLO and CL-LLO constituted either in the Triton X-100 or just in PBS buffer was fractionated on the gradient and analysed. Whereas LLO remained at the bottom of the gradient (Figure 3.7C, iii), CL-LLO was found to float on the gradient (Figure 3.7C iv and vi). The buoyancy of CL-LLO and membrane bound LLO (Figure 3.7Ci) was significantly decreased by the raft-disrupting detergent saponin (Figure 3.7Cii and v). Collectively, these results demonstrate that although LLO and CL-LLO might associate with membrane regions that fulfil the raft criteria of detergent insolubility and low buoyancy, complexing with cholesterol alone, whether in solution or in the cell membranes, is enough to impact such properties onto the toxin.

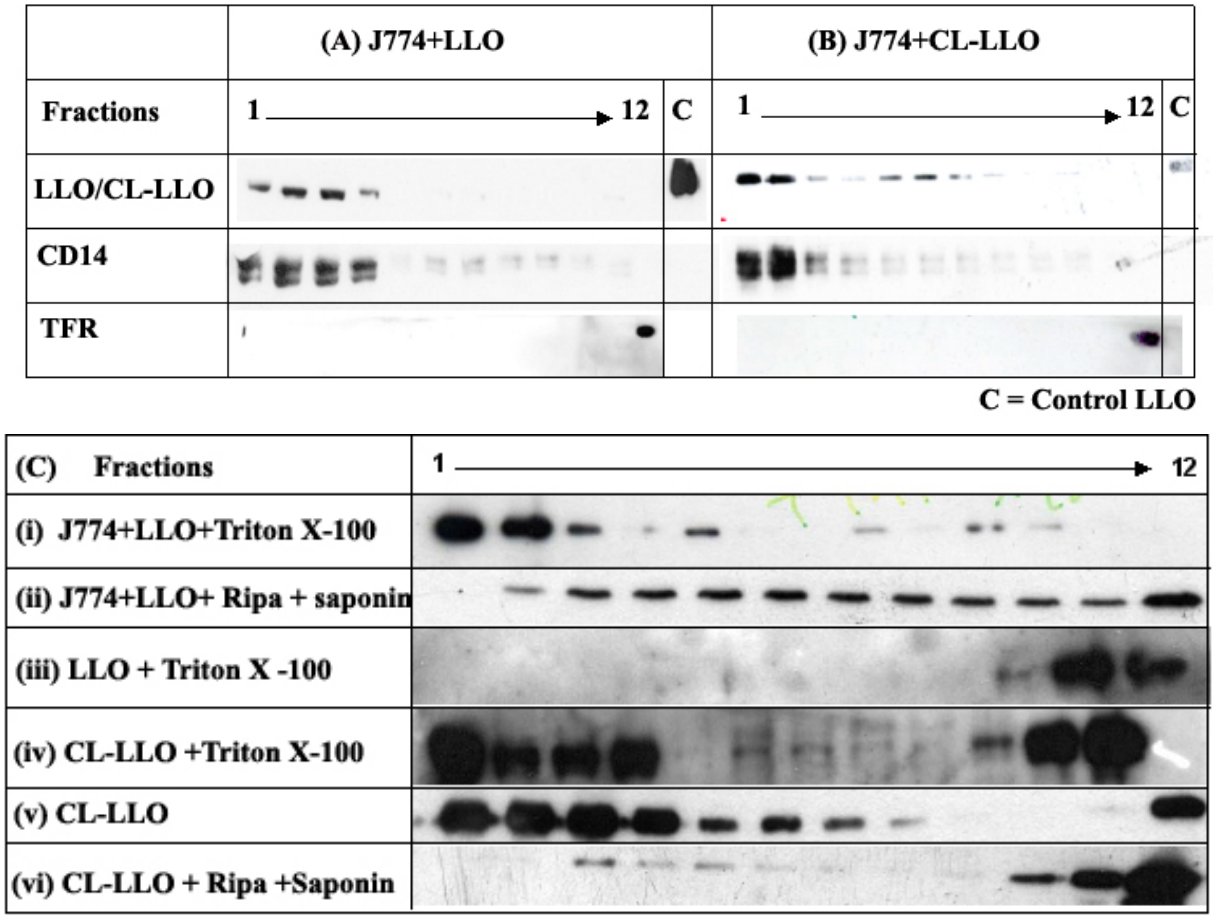

Figure 3.7. Membrane bound LLO and CL-LLO partition into the CD14 enriched Detergent Insoluble Membranes (DRMs). J774 cells treated with LLO **(A)**, or CL-LLO **(B**) for 15 min at room temperature, were solubilized in 1% Triton X-100 on ice. The cell lysates were then fractionated on a 16-40% step sucrose gradient by ultracentrifugation. 12 fractions collected from top to bottom of the gradient (fractions 1 to 12) were analysed by immunoblotting for the presence of LLO, CD14 and the transferrin receptor (TFR). For control, LLO was loaded in the lane marked C (A and **B**). **C:** Binding to cholesterol in solution confers detergent insolubility and buoyancy to LLO. J774 cells treated with LLO were solubilized in 1% Triton X-100 (i), or in a saponin lysis buffer (ii). In parallel, soluble LLO and CL-LLO were also solubilized in either Triton X-100 (iii & iv respectively), saponin lysis buffer (v) or in PBS (vi). All the above preparations were then fractionated and the distribution pattern of LLO/CL-LLO in the sucrose gradient analysed as in A and B.

3.2.3 Effect of LLO on the membrane distribution of raft associated molecules

To corroborate on the interaction of LLO with lipid rafts, immunofluorescence was used to analyse the membrane distribution of a HA tagged LLO (HA-LLO) or its cholesterol-inactivated form (CL-HA-LLO) in relation to described raft associated molecules.

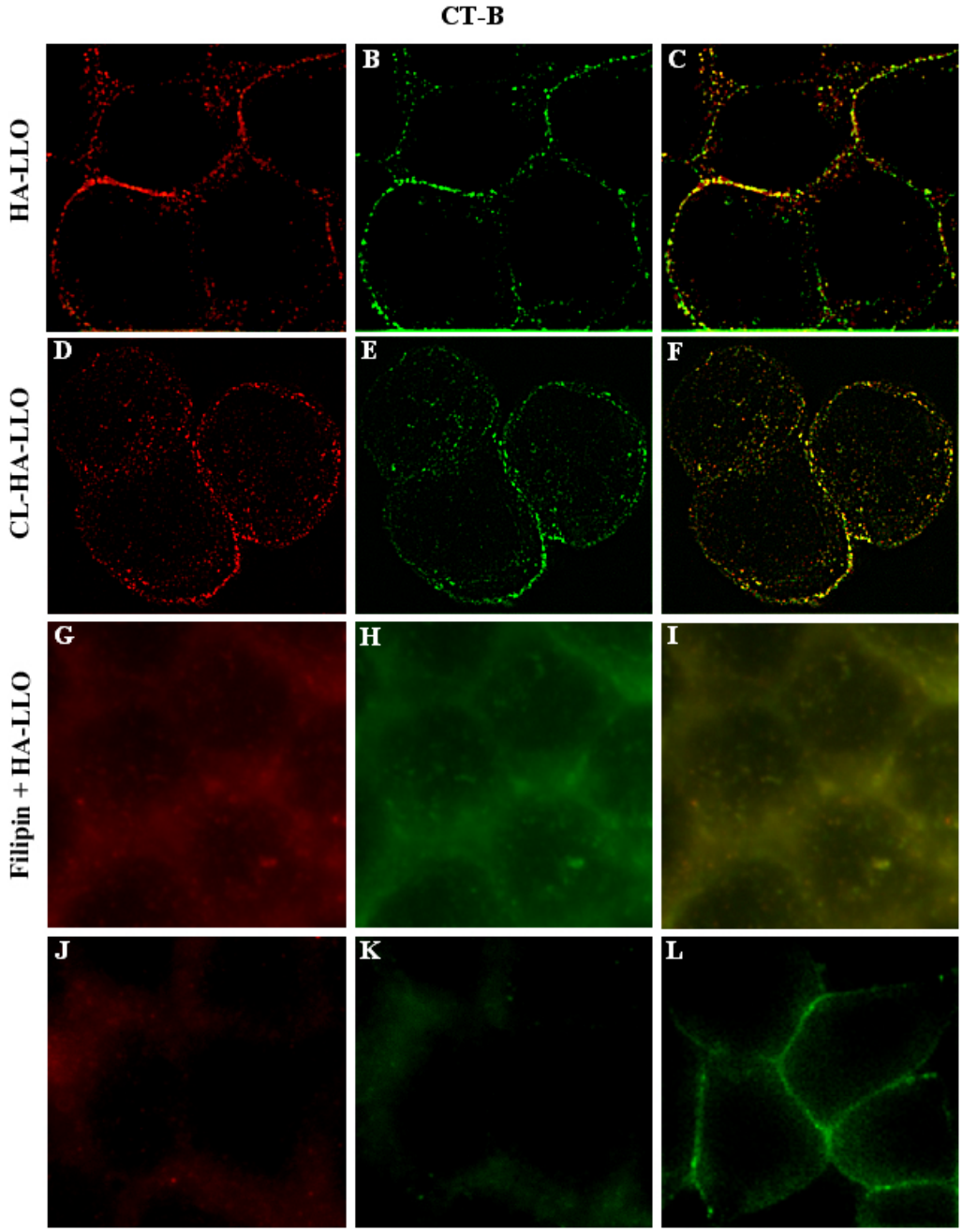

Figure 3.8. Clustering of GM1 by HA-LLO and CL-HA-LLO. J774 cells were treated with HA-LLO (**A-C**) or CL-HA-LLO (**D-F**) at RT and fixed in PFA before being labelled with CT-B. HA-LLO and CL-HA-LLO binding is shown in red and CT-B binding in green, while yellow indicates a merge of the two signals. In **G-I**, cells were first incubated with filipin (100 µg/ml) for 1 h to sequester cholesterol before treatment with HA-LLO and CT-B as in A-C. **J** and **K** show the antibody controls for HA-LLO and CT-B staining, respectively. **L** shows the GM1 distribution on cells at basal conditions i.e. untreated cells fixed before incubation with CT-B.

Cholera toxin subunit B (CT-B), which labels the raft - associated gangliosides GM1, was chosen as a marker. When J774 cells were first incubated with HA-LLO or CL-HA-LLO and fixed with paraformaldehyde (PFA) before labelling with CT-B, GM1 was found in clusters that extensively co-localized with those of HA-LLO or CL-HA-LLO (Figure 3.8A-F). This is in contrast to the uniform GM1 distribution observed on untreated cells (Figure 3.8L), and suggests that such clustering is induced by LLO. Antibody induced redistribution of proteins into patches if they are not properly cross-linked with the fixative has been documented (232). To eliminate such a possibility, two fixation procedures have been used in this study, PFA and the PFA/Ac-MeOH fixation. The PFA/Ac-MeOH fixation procedure has been reported to be very effective in avoiding post staining aggregation (111;233). Here, as will be shown in the next figures, reproducible staining with respect to a dispersed distribution of rafts associated cell surface molecules was obtained with both procedures. One limitation observed with the PFA/Ac-MeOH fixation protocol is a high background staining, especially when using streptavidin based secondary reagents. Therefore, depending on the suitability of reagent combinations, either one of the two procedures has been employed in this study. Figure 3.9 shows the distribution of GM1 on cells fixed with PFA/Ac-MeOH, after HA-LLO treatment. Again, GM1 displays an extensive co-clustering with HA-LLO under this condition.

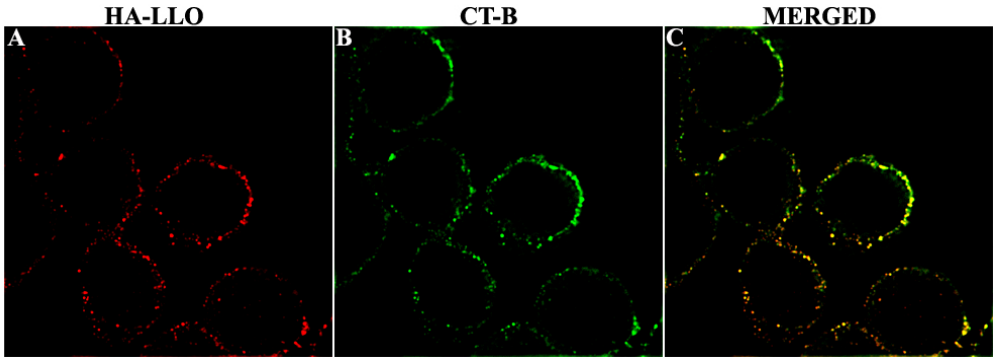

Figure 3.9. Co-clustering of GM1 with HA-LLO on PFA/Ac-MeOH fixed cells. J774 cells treated with HA-LLO were fixed with PFA/Ac-MeOH before labeling the GM1 with CT-B. Bound HA-LLO was revealed using a mouse anti-HA followed by Cy3-goat anti-mouse IgG (**A)** while CT-B was stained using goat anti-CT-B followed by donkey Cy2-donkey anti-goat (**B**).

To determine whether such co-clustering can be generalized to other raft-associated components, plasma membrane distribution of GPI-anchored proteins CD14, CD16 and CD24 on J774 cells after LLO treatment also analysed. Figure 3.10 shows the distribution pattern of HA-LLO, CL-HA-LLO as well as CD14 on J774 cells fixed with PFA post toxin treatment. As predicted, CD14 also exhibits an extensive co-clustering with HA-LLO or CL-HA-LLO (Figure 3.10A-F). Figure 3.10G-I displays the staining of HA-LLO and CD14 on J774 cells that were fixed in PFA before treatment with HA-LLO. No obvious clustering of LLO and CD14 was observed under these conditions. Thus, pre-fixation of membranes interferes with the ability of LLO to aggregate raft components.

To determine the specificity of clustering by LLO, the membrane distribution of HA-LLO and the non-raft marker transferrin receptor (TFR) on J774 cells was also analysed (Figure 3.10J-L). Although the TFR tends to exhibit some patchy distribution even under basal conditions (data not shown), the clear segregation of the LLO clusters from the TFR indicates that LLO neither associates with nor induces clustering of TFR. Similarly, Figure 3.11 shows the distribution pattern of CD14 and TFR in relation to HA-LLO on PFA/Ac-MeOH fixed cells. In this experiment, confocal microscopy was used to visualize and quantify the co-clustering of HA-LLO and CD14. The images shown in Figure 3.11C-E and F-H depict the distribution pattern of HA-LLO and CD14 viewed along the equatorial and polar planes of the same field, respectively. Unlike on untreated cells (Figure 3.11B), CD14 shows an extensive co-clustering with HA-LLO (Figure 3.11C-H) while segregating from the TFR (Figure 3.11J-L), as observed before. The fluorographs shown in Figure 3.11I-M display quantification of the co-localization between the HA-LLO and CD14 (Figure 3.11F-H) as well as TFR (Figure 3.11J-L) respectively. Clustering of other raft associated molecules such as CD16 and CD24 by LLO was also observed (Figure 3.12 and data not shown).

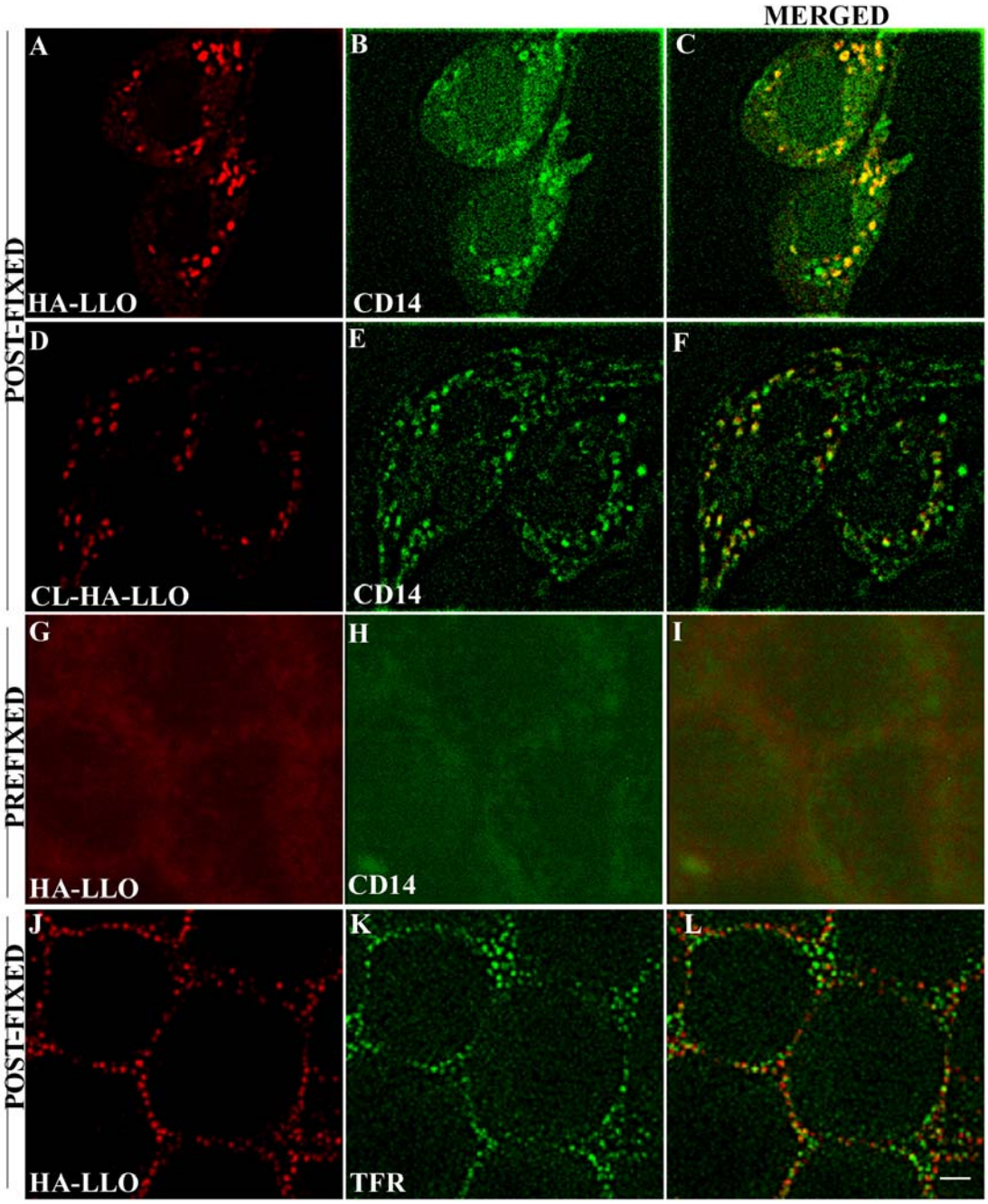

Figure 3.10. HA-LLO and CL-HA-LLO induce clustering of CD14 but not the TFR. The panels **A-C** and **D-F** display cells, first incubated with HA-LLO and CL-HA-LLO, respectively, before fixation. **G-I** shows HA-LLO and CD14 staining on cells fixed before HA-LLO treatment. **J-L** shows the HA-LLO and TFR staining on cells fixed after the HA-LLO treatment.

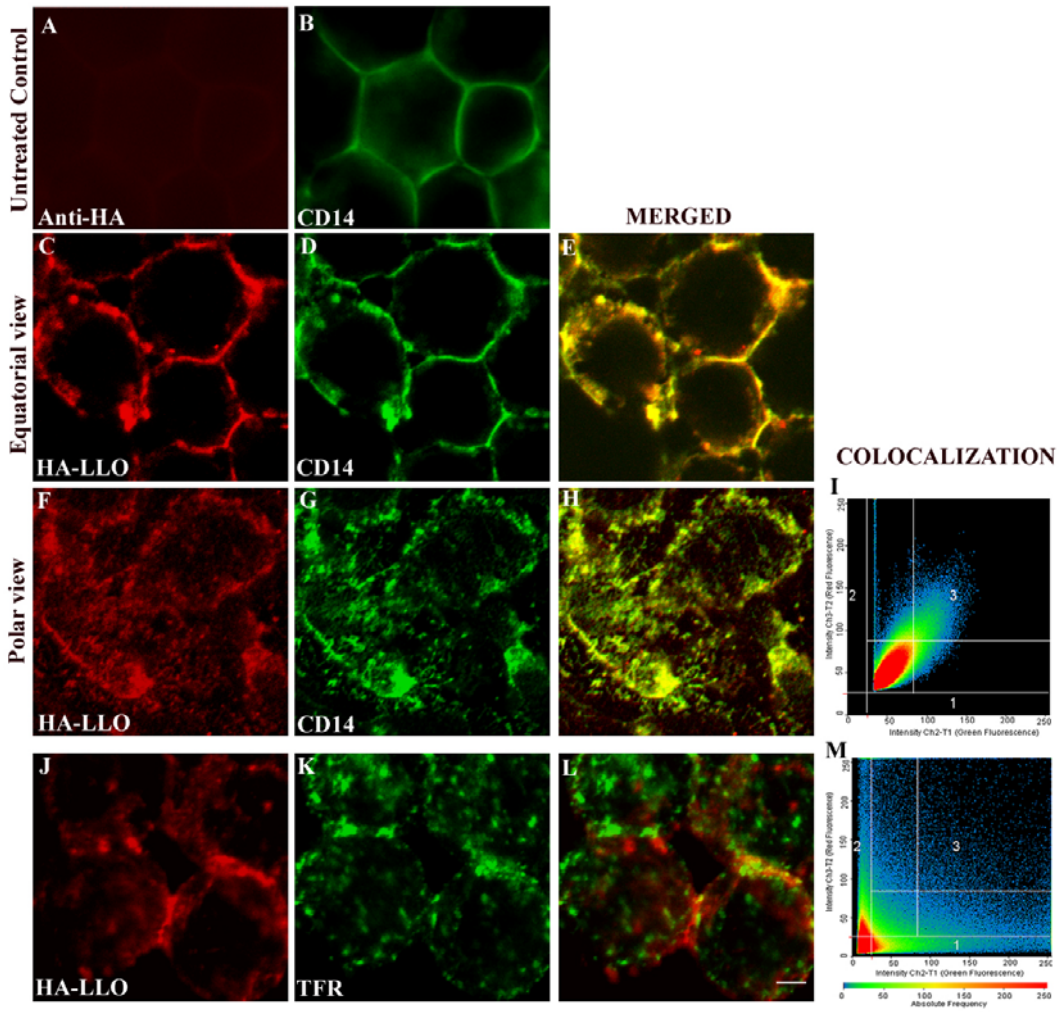

Figure 3.11. Quantification of co-localization between HA-LLO and CD14 or TFR. Cells treated with (or without) HA-LLO and fixed with PFA/Ac-MeOH, were stained for the bound toxin, CD14 as well as TFR before analysis by confocal microscopy. **A** and **B** show the anti-HA and CD14 staining on untreated cells. **C-E** and **F-H** depicts the distribution pattern of HA-LLO and CD14 viewed along the equatorial and polar planes of the same field, respectively. **J-L** shows the HA-LLO and TFR staining. HA-LLO was stained using mouse anti-HA and Cy3-anti-mouse IgG while CD14 was revealed using rat anti-CD14 and alexa488 coupled anti-rat-IgG. The co-localization fluorographs displayed in **I** and **M** show the intensities and scatter pattern of all pixels within the merged images **H** and **L** respectively. Pixels with mostly one fluorescent component are placed along the axes 1 and 2 while the pixels with equal fluorescence intensity from both components (due to co-localization) are placed along the diagonal. Axes 1 and 2, measured green and red fluorescence intensity of pixels on an arbitrary scale from 0 to 250.

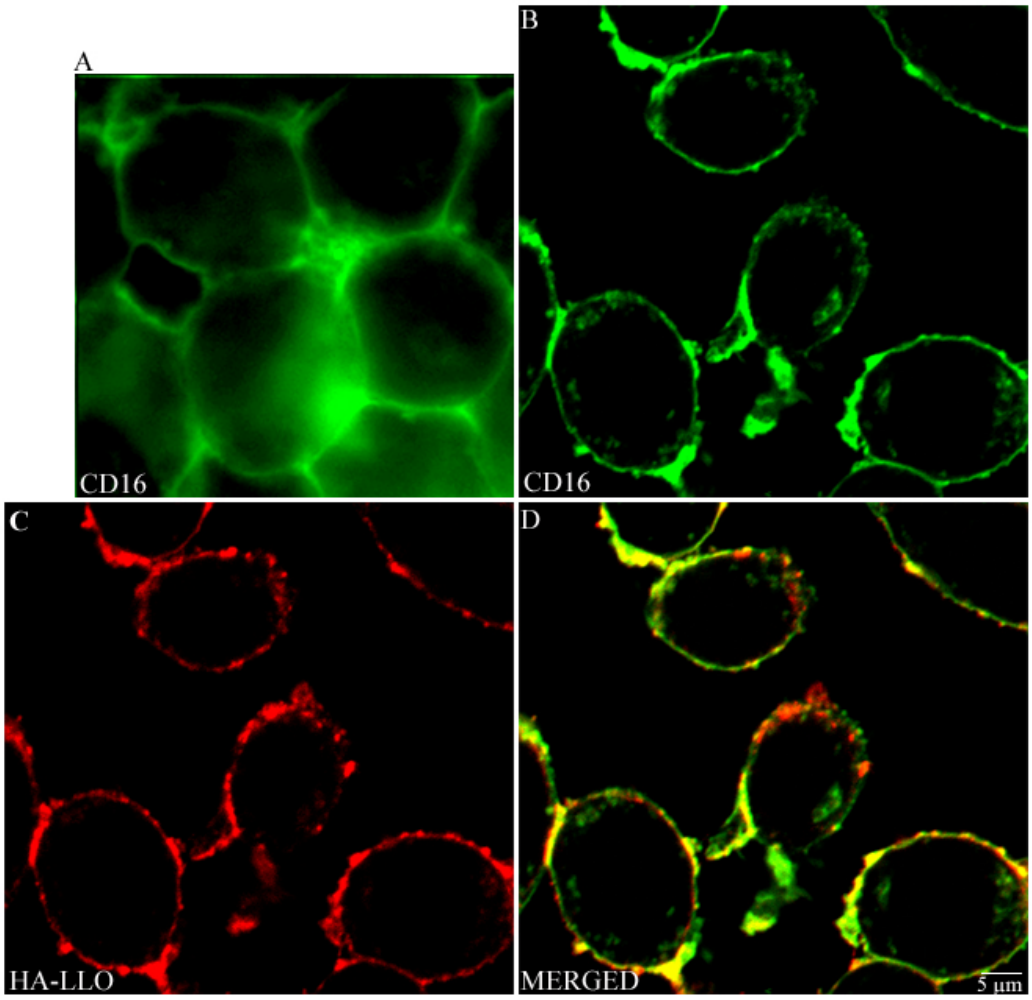

Figure 3.12. HA-LLO induces co-clustering of CD16. Cells treated with HA-LLO were fixed with PFA/Ac-MeOH before staining for bound toxin and CD16. HA-LLO was revealed using a mouse anti-HA followed by Cy3-goat anti-mouse IgG (**B)** while CD16 was stained using a rat anti-CD16 followed by Alexa488-goat anti-rat IgG (**C**). **D** is the merger of **B** and **C** while **A** shows the CD16 staining on untreated cells.

3.2.4 Mechanism of rafts aggregation by LLO

After binding to cholesterol on cell membranes, LLO monomers diffuse laterally to interact with each other to form oligomers. Since clustering of LLO or raft components is not observed under conditions in which lateral diffusion is blocked by prefixing membranes, it was hypothesized that such clustering is due to spontaneous oligomerization of membrane bound LLO monomers.

To test this hypothesis, unfixed or prefixed cells treated with LLO or CL-LLO were analysed for the presence of oligomers by fractionation on sucrose gradients as previously described (66;225). Consistent with the prediction, the preponderance of

LLO as well as CL-LLO bound to unfixed cells was found to be in the high molecular weight oligomeric form (Figure 3.13 C and D). In contrast, LLO bound to prefixed cells remained largely in monomeric form (Figure 3.13E). Thus, the oligomerization of LLO and CL-LLO correlates with the clustering of the raft components by LLO.

The neutralizing monoclonal antibody M344 which recognises a N-terminal epitope of LLO (between position 152-159) was proposed to block LLO oligomerization without inhibiting membrane binding (234).

Figure 3.13. LLO does not oligomerize on prefixed membranes while neutralization with M344 antibody blocks its oligomerization on unfixed membranes. Unfixed (**C-G**), or pre-fixed (E) J774 cells were incubated for 10 min at RT with the following LLO formulations; the active LLO (**C** and **E**), CL-LLO (**D**), LLO neutralized with M344 antibody (LLO+M344) (F) or LLO+M344 additionally treated with cholesterol (CL-LLO+M344). After washing off unbound toxin, cells were solubilized in sodium deoxycholate, before fractionation on a continuous sucrose density gradient by ultracentrifugation. TCA precipitates of eleven equal fractions collected from top to bottom were analysed by immunoblotting for the presence of LLO. LLO monomers and oligomers are defined by their sedimentation behaviour in the gradient. Whereas the higher molecular weight LLO polymers sediment to the bottom of the gradient (fractions 8 -11), the lighter LLO monomers are mainly found at the top (fractions 1-7). Prior to membrane binding LLO and CL-LLO are mainly monomeric (**A** and **B**) but undergoe oligomerization on unfixed (**C** and **D**) but not prefixed (**E**) cell membranes. Pre-incubation of LLO with M344 abrogates its subsequent oligomerization on unfixed membranes (**F**), which is however restored by the additional treatment with cholesterol (**G**). The LLO found in the high molecular fraction in A might be due spontaneous aggregations in solution akin to the phenomenon reported for SLO (235). The LLO found in the high molecular weight fraction in F is most likely due to incomplete neutralization by the antibody M344.

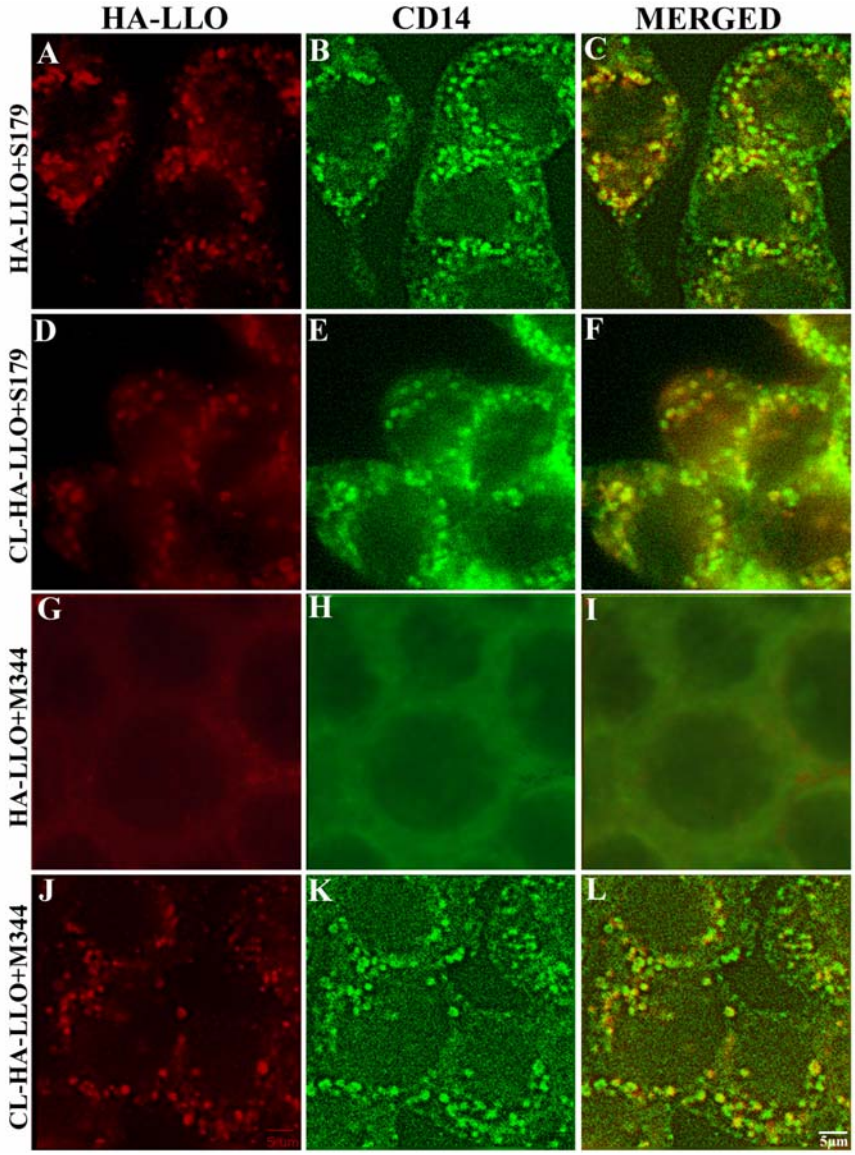

Figure 3.14. The neutralizing antibody M344 block rafts aggregation by HA-LLO but not by CL-HA-LLO. J774 cells were incubated at RT with 1 µg/ml of the following LLO formulations; HA-LLO pre-incubated with the non-neutralizing S179 antibody (HA-LLO+S179) (**A-C**), HA-LLO+S179 additionally treated with cholesterol (CL-HALLO+S179) (**D-F**), HA-LLO neutralized with M344 (HA-LLO+M344) (**G-I**) or HA-LLO+M344 treated with cholesterol in addition (CL-HALLO+M344) (**J-L**). After washing off unbound toxin, cells were fixed with PFA before stained for the bound HA-LLO (red) and CD14 (green). HA-LLO staining was done using a biotinylated anti-HA followed by Cy3-streptavidin.CD14 was stained using a directly labeled FITC-anti-CD14. While brigher fluorescence signals due to local concentration of both molecules in the clusters can be observed where oligomerization occurred, dimmer fluorescence signals are obtained from the diffused LLO monomers and CD14 on cells despite the equal amount of bound LLO (flow cytometry data not shown). Scale bar, 5 µm.

To explicitly demonstrate the role of LLO oligomerization in the aggregation of rafts, whether the M344 antibody could abrogate the co-clustering of rafts components by LLO was examined. To that end, HA-LLO or CL-HA-LLO were pre-incubated with the

neutralizing or non-neutralizing antibody before treating cells and evaluating the clustering of CD14. First, controls using the non-neutralizing antibody S179 show the aggregation of CD14 by HA-LLO and CL-HA-LLO as observed before (Figure 3.14 A-F).

In contrast, aggregation of CD14 was substantially inhibited when HA-LLO was neutralized with M344 (Figure 3.14G-I). Surprisingly, HA-LLO neutralized by M344 could regain the ability to aggregate CD14 if additionally treated with cholesterol (Figure 3.14J-L). Analysis of extracts of cells treated with HA-LLO+M344 or CL-HA-LLO+M344 for LLO oligomers confirmed that the M344 antibody indeed blocks oligomerization, which can nonetheless be restored by cholesterol treatment (Figure 3.13F and G).

3.2.5 LLO induces tyrosine phosphorylation in a raft aggregation dependent manner

What is the functional significance of rafts aggregation by LLO? To address this question, the induction of tyrosine phosphorylation by LLO in J774 cells was evaluated. By immunoblotting it was observed that both LLO and CL-LLO induce tyrosine phosphorylation (112). Similarly, when analysed by immunofluorescence it was found that both HA-LLO and CL-HA-LLO could still induce tyrosine phosphorylation in the presence of the non-neutralizing isotype control antibody S179 (Figure 3.15A-F). In contrast, but in agreement with oligomerization and rafts aggregation data (Figure 3.13 and Figure 3.14), neutralization with M344 abrogates the ability of HA-LLO to induce tyrosine phosphorylation (Figure 3.15G-I). This can however be restored by the additional treatment of the pre-formed LLO+M344 complexes with cholesterol (Figure 3.15J-L), thus, demonstrating that the co-clustering of raft components results in the induction of signals due to the oligomerization of membrane bound LLO monomers.

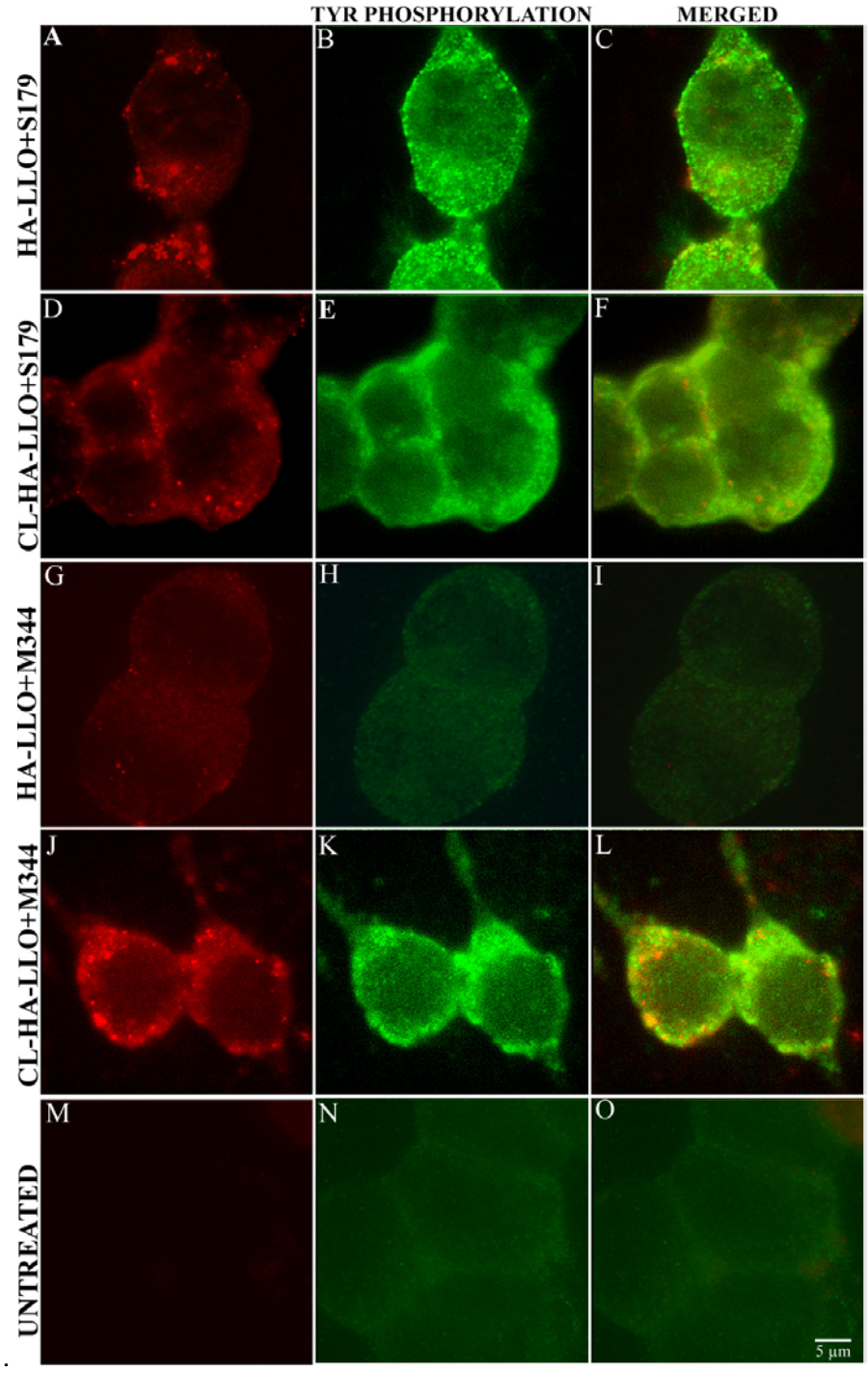

Figure 3.15. The antibody M344 inhibits activation of tyrosine phosphorylation by HA-LLO but not CL-HALLO. J774 cells were incubated for 5 min at 37° C with 0.25 µg/ml of HA-LLO+S179 (A-C), CL-LLO+S179 (D-F), HA-LLO+M344 (G-I), or CL-HA-LLO+M344 (J-L). After washing, and fixation with PFA, cells were stained for surface bound toxin (red) after which they were permeabilized in 0.1% Triton X-100 then stained for phosphotyrosine (middle column). M-O shows the respective staining in the unstimulated control cells. Scale bar, 5 µm.

3.2.6 Activation of Lyn and Syk by LLO

The src family kinase Lyn is anchored to the inner leaflet of the plasma membrane by way of myristate and palmitate chains that targets it to lipid rafts. To further elucidate the role of rafts aggregation in the induction of signalling whether Lyn also undergoes redistribution upon LLO treatment was investigated. Figure 3.16A shows that under basal conditions Lyn is uniformly distributed on the cell membrane. Upon treatment with HA-LLO an appreciable redistribution of Lyn into the LLO patches could be observed (Figure 3.16B-D).

Whether LLO triggers redistribution of the cytoplasmic kinase Syk was also investigated. In this case however, no significant co-localisation of Syk with the LLO patches was observed (data not shown).

Next the tyrosine phosphorylation status of these two kinases in response to LLO stimulation was investigated. J774 cells stimulated for the indicated durations (Figure 3.16E) were lysed with cold 1% TritonX-100. The DRM and soluble materials (contained in the pellet and supernatant, respectively) were separated by centrifugation. Subsequently, representative samples of the DRM and soluble fractions were subjected to immunoprecipitation using an anti-phosphotyrosine antibody. The immunoprecipitates were then immunoblotted and developed with anti-Lyn and anti-Syk antibodies. As evident from Figure 3.16E, both Lyn and Syk undergo a rapid tyrosine phosphorylation upon stimulation with LLO. As expected, Lyn was mainly enriched in the DRM while Syk was predominantly localised in the soluble fraction. A modest but transient recruitment of Syk to the DRM could however be observed (Figure 3.16E).

It is noteworthy to mention that the tyrosine phosphorylation phase of these kinases upon LLO stimulation is quite transient. Prolonged stimulation (longer that 15 min) resulted in diminishing the phosphorylated form of these kinases (data not shown). This implies that, although activation of tyrosine kinases in macrophages by LLO might dominate the proximal signalling events induced, LLO also activates downstream negative regulators.

In summary, the results presented show how, by aggregation of rafts via oligomerization, LLO causes the signal induction in target host cells.

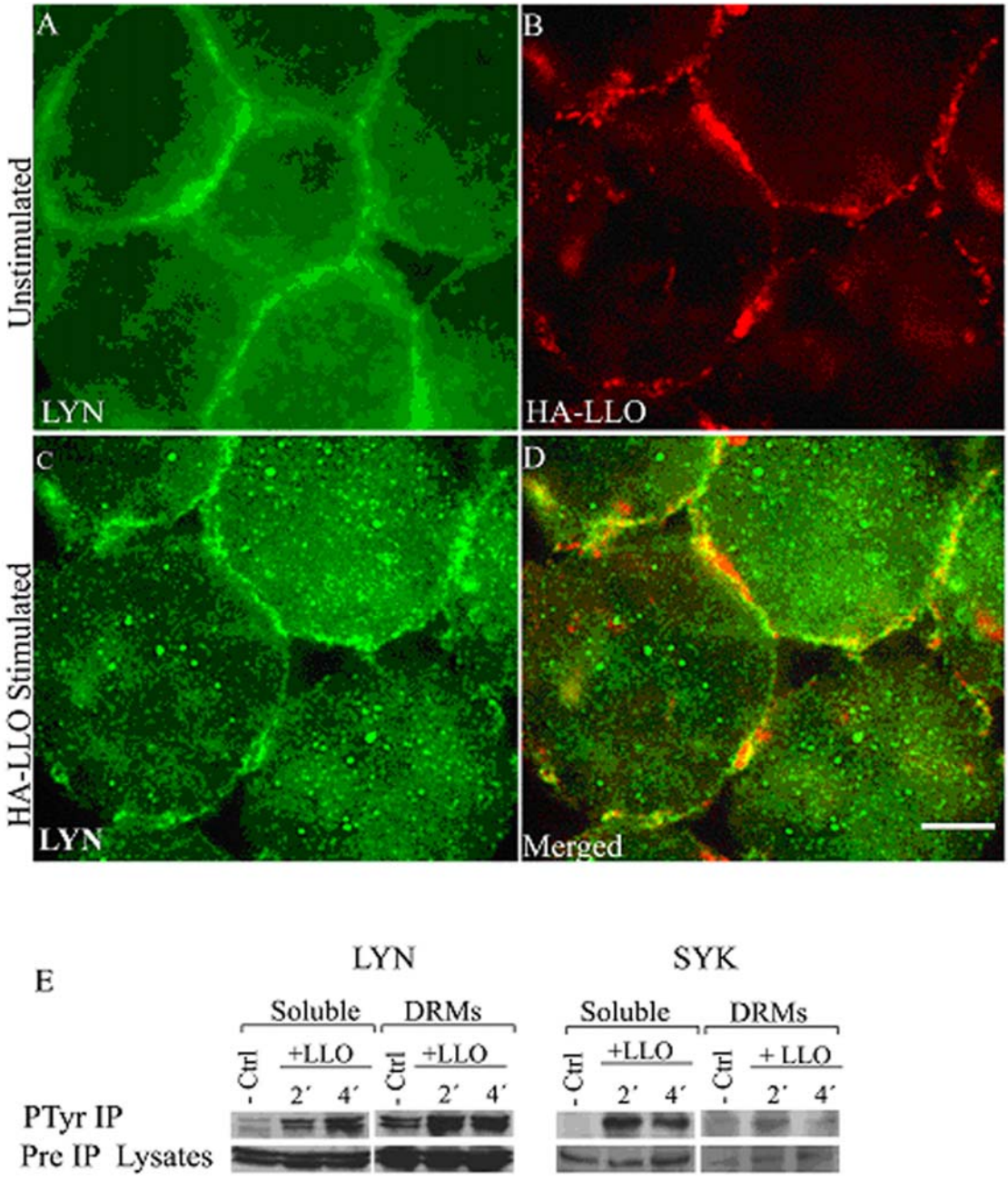

Figure 3.16. Redistribution of Lyn into patches upon LLO treatment is accompanied by the activation of Lyn and Syk. Untreated (A) or HA-LLO treated cells (B-D) were fixed with PFA/Ac-MeOH before staining for Lyn and bound toxin. E: Cells treated with HA-LLO for 2 and 4 min were lysed in ice cold 1% Triton-X 100. After centrifugation (20,000 g for 30 min), the supernatant (soluble) and the pellet (DRM) fractions were subjected to immunoprecipitation (IP) using the anti-phosphotyrosine antibody (PY99). Subsequently, the IPs were immunoblotted and developed with anti-Lyn and-Syk antibodies. Lyn and Syk immunoblots of the pre-IP lysates are shown (bottom row). Scale bar, 5µm.

3.3 Role of mast cells in the control of Listeriosis

As discussed in the introduction, mast cells are the first cell types in which evidence for the involvement of rafts in signaling was demonstrated. Recognition of antigen by specific IgE bound to FcεRI leads to the aggregation of FcεRI and hence its recruitment into rafts where signalling cascades that culminate in gene transcription and degranulation are initiated (140). As shown in section 3.1, calcium dependent processes regulate mast cells functions such as the spontaneous release of inflammatory mediators. Given that LLO triggers signalling in host cells via rafts as well as calcium signalling, mast cells were considered an ideal cell type for analysing the functional significance of signal induction by LLO during *Listeria* infection.

As mentioned before, mast cells have largely been studied in the context of allergic diseases. However, their role in the control of pathogens is now well established (28;32;226;236;237). As partly outlined before, mast cells are endowed with several properties that place them in the frontline of host defences against invading pathogens. (1) They have a wide tissue distribution especially at the host-environment interfaces such as the skin, airways and gastrointestinal tract, where pathogens, allergens and other environmental agents are frequently encountered (23). (2) In addition to *de novo* synthesis, mast cells have stored presynthesized inflammatory mediators thus being the most readily available source of such mediators during the early course of infection (238). (3) Mast cells can be activated by many different stimuli, acting via several signalling pathways, during innate and acquired immune responses (239). (4) They are long lived and can re-enter cell cycle and proliferate locally (23;239).

Although a ready source of mediators and potentially being among the first cell types with which bacteria come into contact with, mast cells have so far not been investigated in the context of listeriosis. Thus, to assess the functional outcome of signals induced by LLO as well as live bacteria, *in vitro* and *in vivo*, mast cells were tested for their influence in the course infection by *L. monocytogenes.*

3.3.1 *L. monocytogenes* induces cytokine and chemokine transcription via LLO dependent and independent mechanisms

In section 3.1.2 it was shown that both LLO and wild-type *L. monocytogenes* do induce degranulation as well as *de novo* synthesis of proinflammatory mediators such as TNF-α. To investigate further, the following questions with respect to the induction of cytokine and chemokine synthesis by LLO and whole bacteria were considered. (1) Does cholesterol inactivated LLO (CL-LLO) which does not form pores but aggregates rafts also induce cytokine /chemokine synthesis? (2) Is cytokine/chemokine synthesis by *L. monocytogenes* solely due to LLO or can other bacterial products induce such a response? (3) Since LLO also promotes the intracellular life cycle of *L. monocytogenes*, is the invasion of the cytosol by the bacteria required for the induction of the above responses? To address the above questions, bone marrow derived mast cells (BMMCs) were evaluated by RT-PCR for mRNA expression of TNF-α, Monocyte chemotactic protein-1 (MCP-1) and Macrophage inflammatory protein-1alpha (MIP-1α) in response to LLO, CL-LLO, wild-type *L. monocytogenes* (WTL.m), heat killed WTL.m (HKLWT) as well as the avirulent LLO deficient *L. monocytogenes* mutant strain (ΔhlyL.m) or HKLΔhly.

As shown before, the transcription of TNF- α was found to be induced by LLO but not by CL-LLO (Figure 3.17). Both, MIP-1α and MCP-1, genes were however found to be induced by LLO and CL-LLO suggesting that while the transcriptional activation of TNF- α largely dependents on the pore forming activity of LLO, signals induced by LLO via the non-pore dependent mechanisms are sufficient for the transcriptional activation of the MIP-1α and MCP-1 genes (Figure 3.17).

In addition, both the WTL.m and ΔhlyL.m could induce a comparable transcription of these genes suggesting they can also be induced by listerial components independent of LLO. Interestingly, the TNF-α. and MIP-1α genes could also be activated by the HKL preparations of WTL.m and ΔhlyL.m while MCP-1 apparently requires either LLO or viable *Listeria.* This indicated that neither LLO nor the invasion of the cytosol by the bacteria is essential for TNF-α and MIP-1α genes activation (Figure 3.17). Taken together, the data show that although LLO can directly induce the transcription of TNF-α, MIP-1α and MCP-1, such a response can also be induced

by other listerial products without necessarily infecting the cell. In fact, when BMMCs exposed for 3 h (the equivalent duration as for the above samples) to WTL.m and ΔhlyL.m were analysed by electron microscopy, hardly any bacteria were found inside the cell. Despite, bacteria adhered to the cell surface (Figure 3.18). This is in stark contrast to the professional phagocytic cells J774 cells exposed to *Listeria* at the same multiplicity of infection (MOI) (data not shown).

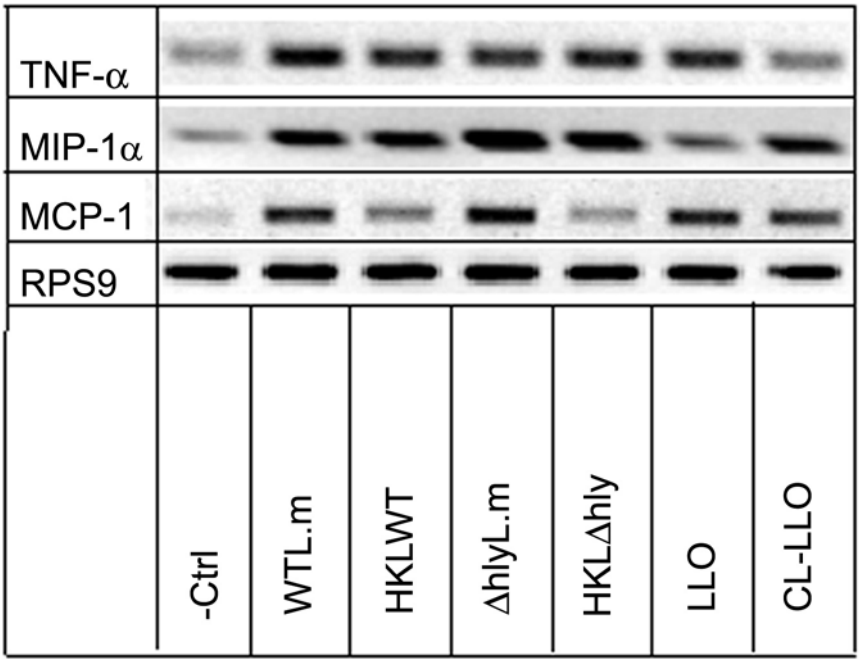

Figure 3.17. *L. monocytogenes* induces cytokine and chemokine transcription via LLO dependent and independent mechanisms. mRNA of TNF-α, MIP-1α, MCP-1 and the house keeping gene RPS9 in BMMCs at 3 h after exposure to the control medium (-Ctrl), WTL.m, HKLWT, ΔhlyL.m, HKLΔhly, LLO or CL-LLO). Incubation of cells was done at a multiplicity of infection (bacteria : cell) of 100 while the predetermined sublytic LLO /CL-LLO concentration of 0.25 µg/ml was used.

Noteworthy to mention also, there was no difference between the WTL.m and ΔhlyL.m in their adherence to the mast cells surface. Therefore, although mast cells are known to have some phagocytic function (240;241), their phagocytic efficiency towards *L. monocytogenes* appears to be extremely low. By chance or design, fortunately, entry into these cells seems not be a requisite for the induction of cytokines and chemokines as demonstrated by the fact that they can be induced by HKLΔhly (Figure 3.17).

3.3.2 *L. monocytogenes* induces recruitment of neutrophils into the peritoneum via LLO dependent and independent mechanisms

The early inflammatory response to infection with *L. monocytogenes* is characterized by the recruitment of granulocytes and monocytes to the site of infection - an essential requirement for control of infection. TNF- α, MCP-1 and MIP-1 α are among the key proinflammatory mediators, responsible for the attraction of such inflammatory cells to the sites of infection (15;16;19;242;243).

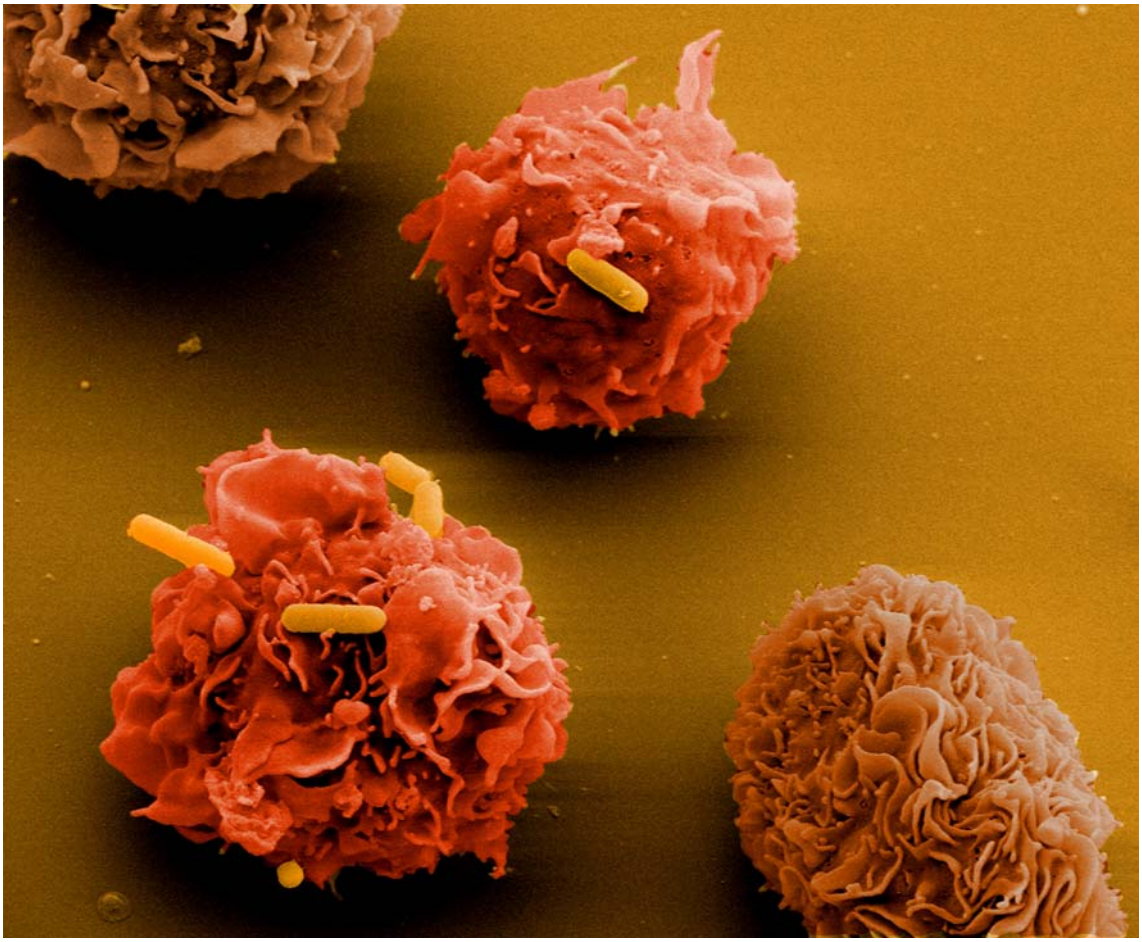

Figure 3.18. Scanning electron micrograph of BMMCs incubated with ΔhlyL.m for 3 h.

Thus, to evaluate the functional outcome of cytokine / chemokine induction, various preparations of *L. monocytogenes* and LLO were injected into the peritoneum of BALB/c mice. 1-3 hours later the peritoneal exudates were analysed for the recruitment of inflammatory cells.

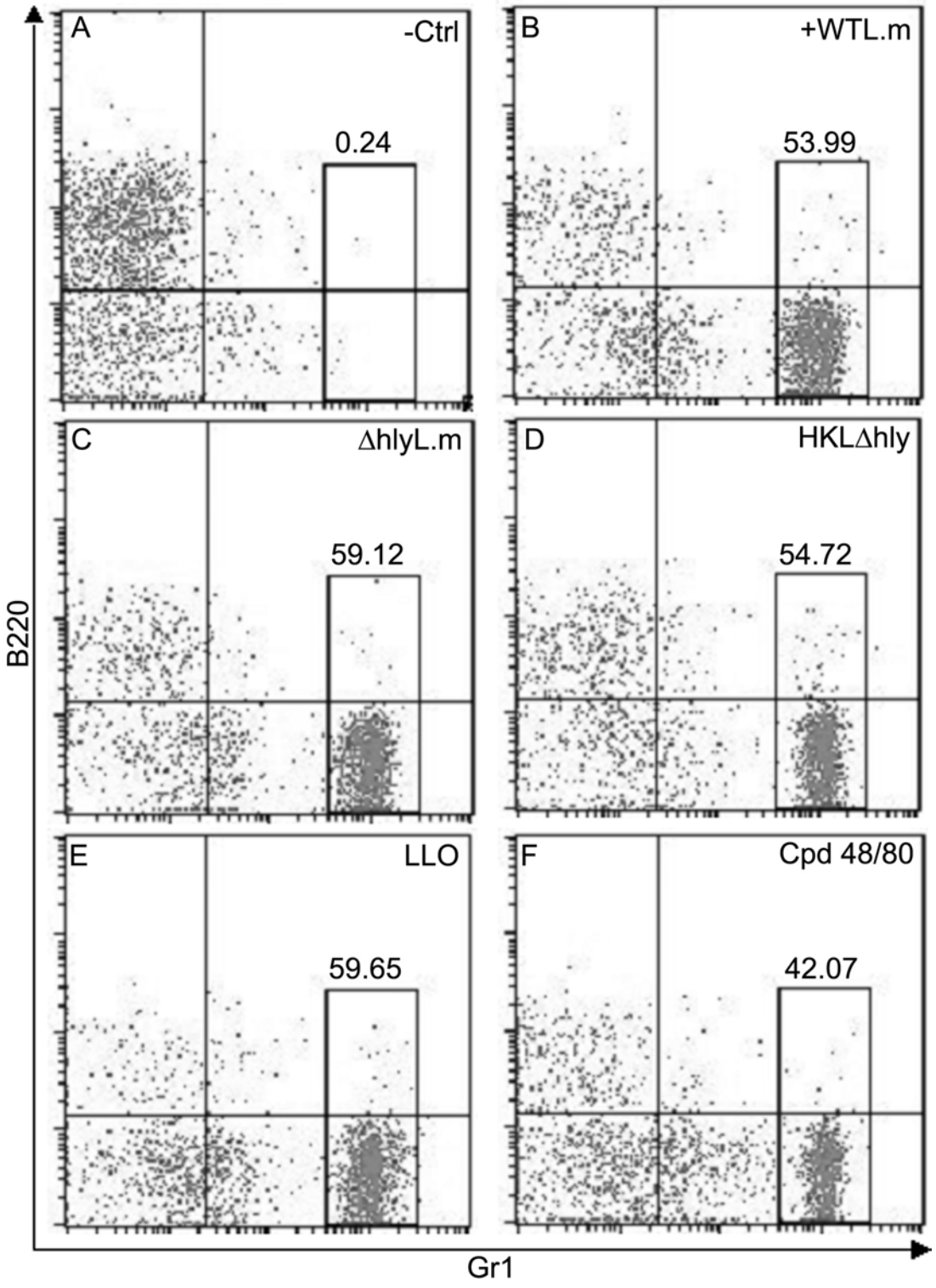

Figure 3.19. *L. monocytogenes* induces recruitment of neutrophils into the peritoneum via LLO dependent and independent mechanisms. Mice were injected intraperitoneally with either of the following: medium control, LLO (0.5 μg), Cpd 40/80 (30 μg) or 1X10^5 of WTL.m, ΔhlyL.m and HKLΔhly. After 3 h peritoneal exudate cells were stained with FITC-anti-Gr1 and APC-anti-B220 and assessed by flow cytometry. Numbers represent the percentage of total cells found in the neutrophil (Gr1$^+$) gate.

Of the cell types analysed, neutrophils - an inflammatory cell type that plays a critical role in reducing bacterial burden in several organs (244) showed the most dramatic influx into the peritoneal cavity during this early period. Consistent with the induction of proinflammatory cytokines and chemokines, this response could be elicited by LLO, as well as live and HKLWT and ΔhlyL.m (Figure 3.19A-E and data not shown).

3.3.3 Mast cells significantly contribute to the recruitment of neutrophils by LLO and *L. monocytogenes*

Due to the pleiotropic effects of LLO and bacteria on host cells, as per the above experimental set-up, it was difficult to estimate the specific contribution of mast cells in the recruitment of neutrophils into the peritoneum. Thus, to specifically evaluate the role of mast cells activation, first, whether the mast cell specific activator compound 48/80 (Cpd 48/80) could also attract neutrophils was tested. Indeed as shown in Figure 3.19F, injection of Cpd 48/80 into the peritoneum induced a robust influx of neutrophils, indicating that mast cells activation by a mast cell specific stimulus was sufficient to induce the peritoneal influx of neutrophils in the peritonitis model employed.

To definitively evaluate the specific contribution of mast cells, neutrophil recruitment was tested in mice depleted of mast cells by injecting the anti-c-Kit monoclonal antibody as previously described (223). Administration of the anti-c-Kit antibody as compared to a control antibody led to the depletion of over 90% of mast cells by 24 hours post intraperitonial injection (Figure 3.20A-B).

When mice were intraperitonially challenged with live WTL.m, mice depleted of mast cells showed a highly diminished capacity to recruit neutrophils compared to control mice (Figure 3.21A-D). This indicates that mast cells are definitively involved in the earliest anti-*Listeria* innate host reactions characterized by the influx of neutrophils to the site of infection.

3.3.4 Accumulation of TNF-α in the peritoneal cavity of mice infected intraperitoneally with *L. monocytogenes*

Next, an attempt was made to determine the mast cell product/s that regulated the recruitment of neutrophils. Focus was put on TNF-α. TNF-α was considered as a prime candidate for at least two reasons. (1) TNF-α is a known potent mediator of

neutrophils attraction to sites of inflammation (28). (2) As mentioned earlier, TNF- α is prestored in mast cell granules and is therefore spontaneously released when mast cells are exposed to bacteria or bacterial products such as LLO (as shown in section 3.1.2). To determine whether mast cell activation by *Listeria* also leads to release of TNF-α *in vivo*, TNF-α concentration in the peritoneal cavity of normal and mast cell depleted mice was measured 3 hours after bacterial challenge.

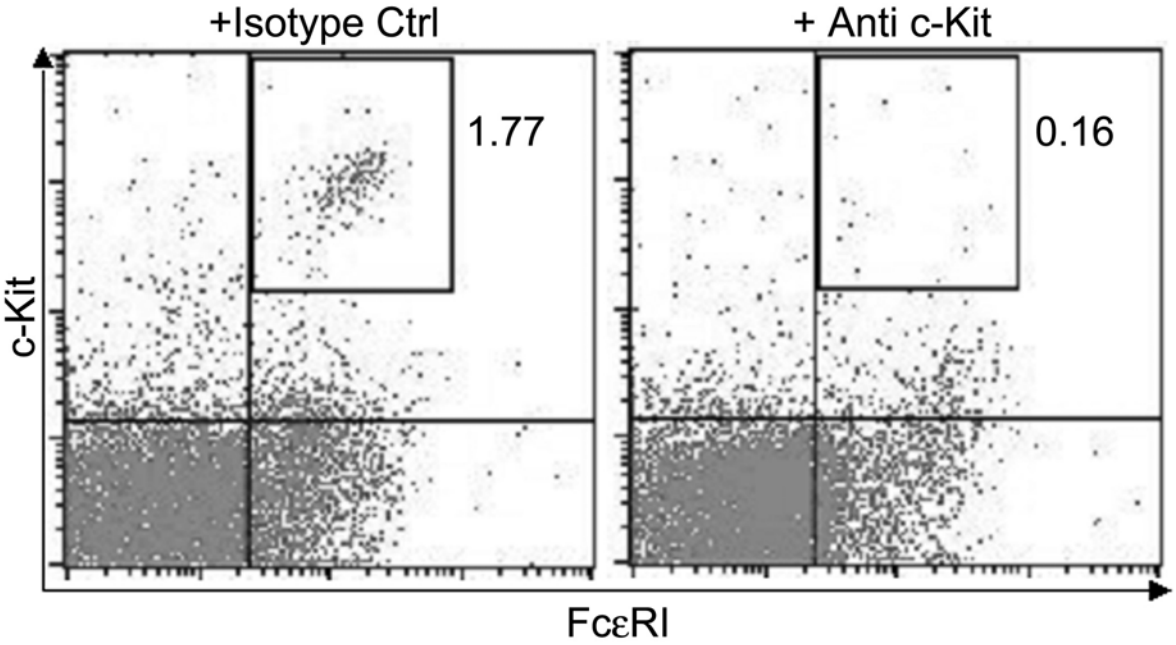

Figure 3.20. Depletion of mast cells in mice using anti-c-Kit antibody. 24 h after intraperitoneal injection of an isotype control antibody (**A**) or the mast cell-depleting antibody anti-c-Kit (**B**), mice were sacrificed and the peritoneal exudate cells stained with FITC-anti-FcεRI and APC-anti-c-Kit (mast cell surface markers) then assesed by flow cytometry. Mice injected with anti-c-Kit antibody (**B**) contained less than 10% of those injected with isotype control (**A**).

As shown in Figure 3.22, a significantly higher concentration of TNF-α was found in the peritoneal exudates of infected mice. Consistent with a requirement for mast cells, diminished TNF-α levels were detected in mast cell-depleted mice (Figure 3.22). This strongly suggests that mast cells are the major source of the TNF-α released in the very early stages of *Listeria* infection.

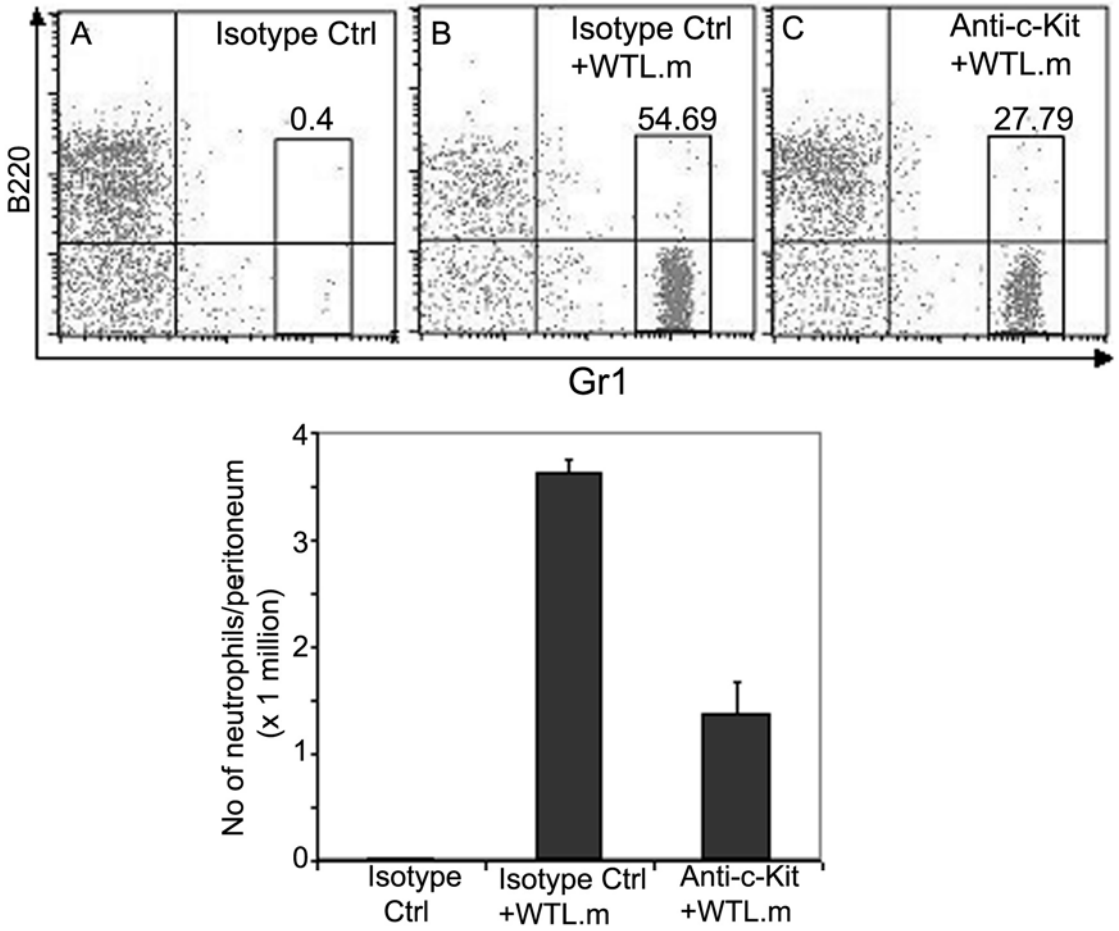

Figure 3.21. Mast cells significantly contribute to the intraperitoneal recruitment of neutrophils by LLO and *L. monocytogenes*. 24h after injection of the control (**A** & **B**) or the anti-c-Kit (**C**) antibodies, mice were infected with $1x10^5$ WTL.m intraperitoneally. 3 h after infection the recruitment of neutrophils into the peritoneum was assessed by staining peritoneal exudate cells with FITC-anti-Gr1 and APC-anti-B220. Each of the stainings shown are representative of peritoneal exudates of three mice. The absolute numbers of neutrophils in the peritoneum (three mice per group) are shown in **D**.

Since mast cell dependent recruitment of neutrophils into the peritoneum appeared to correlate well with accumulation of TNF-α, whether TNF-α alone in the absence of *Listeria* infection could elicite a similar response was tested. Administration of TNF-α into the peritoneum of mice led to a rapid influx of neutrophils into the peritoneum, quite comparable to that triggered by administration of bacteria (Figure 3.23A-C). Taken together the above results show that the release of TNF-α by mast cells following activation by *Listeria* leads to the recruitment of neutrophils a major mediator of innate immunity.

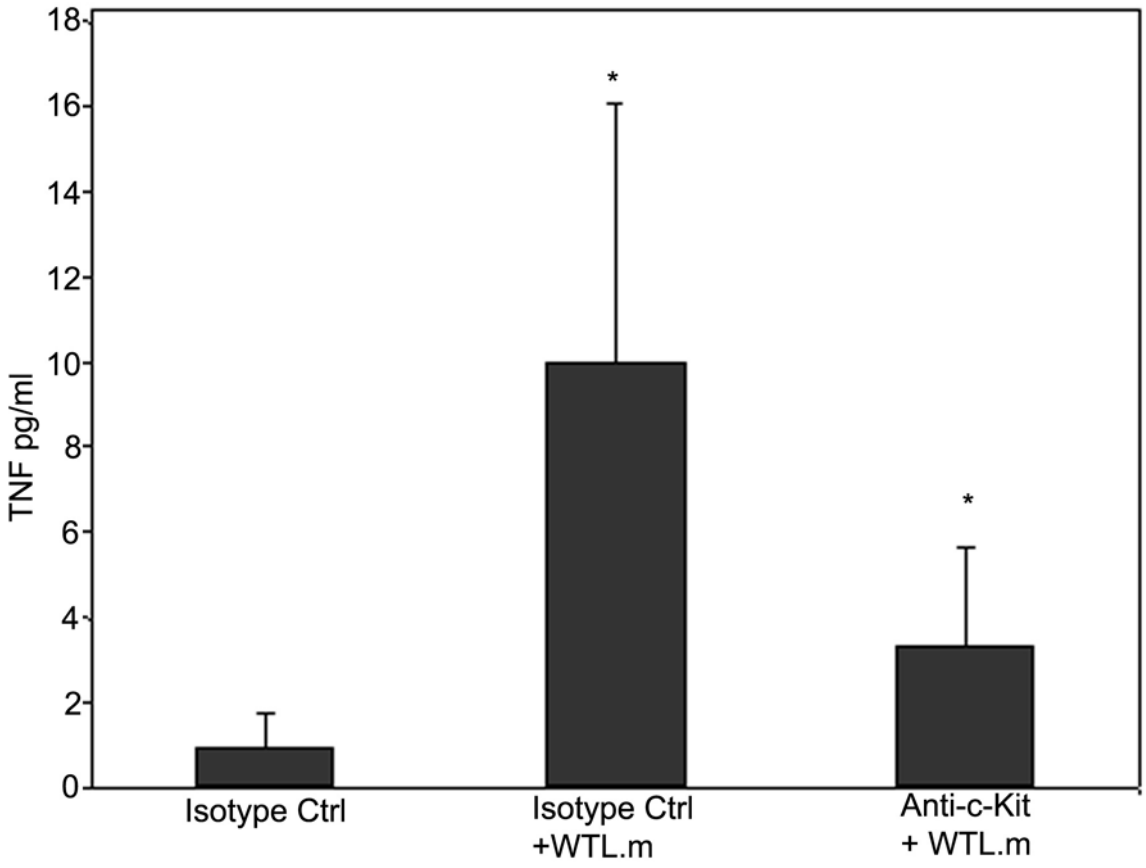

Figure 3.22. *Listeria* induces accumulation of TNF in the peritoneal cavity in a mast cell dependent manner. Mice injected with an isotype control antibody or depleted of mast cells using the anti-c-Kit antibody were infected or not with $1x10^5$ WTL.m intraperitoneally. After 3 h, mice were sacrificed and the peritoneum flushed with 10 ml of tissue culture medium. The washouts were then analyzed for the concentration of TNF. The bars represent the mean and standard deviation of three individually analyzed mice per group (* $p<0.05$).

3.3.5 Mast cell dependent recruitment of neutrophils is required for listerial clearance

The recruitment of neutrophils plays an important role in containing the bacteria at the site of infection hence curbing dissemination to other tissues (244). Accordingly, when neutrophils were depleted using the anti-granulocyte monoclonal antibody RB6-8C5, mice showed a profound deficiency in their innate immune response with the bacterial burden in the liver and spleen of such mice 2 days post infection. Compared to the undepleted controls, over 500 fold more *Listeria* organisms were recovered in the mice injected with RB6-8C5 antibody (Figure 3.24A-B).

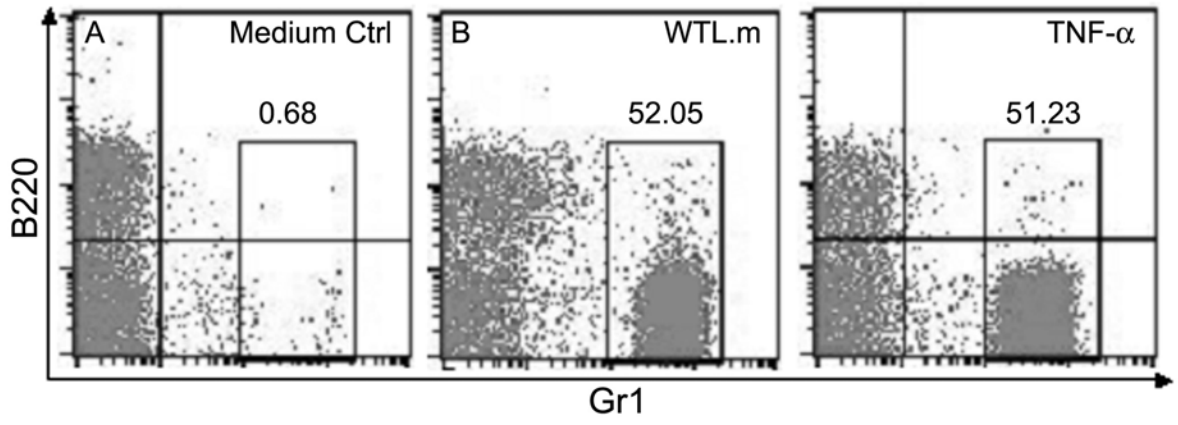

Figure 3.23. TNF-α induces the recruitment of neutrophils into the peritoneum of mice. Mice were treated intraperitoneally with either 200 pg TNF-α, $1x10^5$ WTL.m or the control medium. After 3 h the peritoneal exudate cells were stained with FITC-anti-Gr1 and APC-anti-B220 to assess the recruitment of neutrophils.

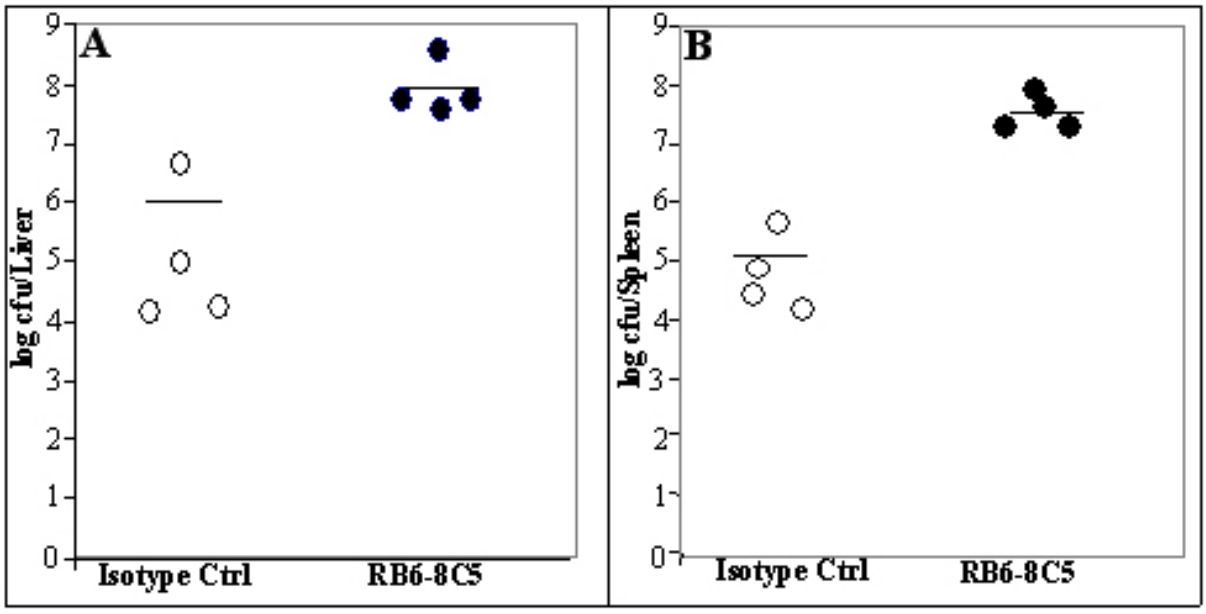

Figure 3.24. Neutrophils play an essential role in the resolution of *L. monocytogenes* infection. 24 h after intraperitoneally administering an isotype control antibody or the neutrophil depleting RB6-8C5 antibody, mice were challenged with $1x10^5$ WTL.m via the intraperitoneal route. 3 days post infection mice were sacrificed and bacterial burden of the liver (**A**) and spleen (**B**) was determined. Circles represent individual mice, and bars represent geometric mean CFU/organ.

In view of the fact that the recruitment of neutrophils to the site of infection was mast cells dependent, the next question was whether depletion of mast cells would also impair the ability of mice to combat *Listeria* infection. For that, mice were injected with either the anti-c-Kit antibody or an isotype control antibody. 24 h later they were

challenged intraperitoneally with *L. monocytogenes*. When analysed 3 days post infection, the bacterial burden in the livers and spleens of mast cells-depleted mice was found to be approx 200 fold and 500 fold, respectively that of mice injected with the control antibody (Figure 3.25A-B).

Interestingly, the difference in bacterial burden in the peritoneum, between the control and mast cell depleted mice was very minimal (Figure 3.25C). Judged by the low cfus (colony forming units), the results show that the peritoneum although the site of bacterial inoculation is not the optimal organ for the replication of *L. monocytogenes,* rather the bacteria disseminate quickly to their major target organs - the spleen and liver.

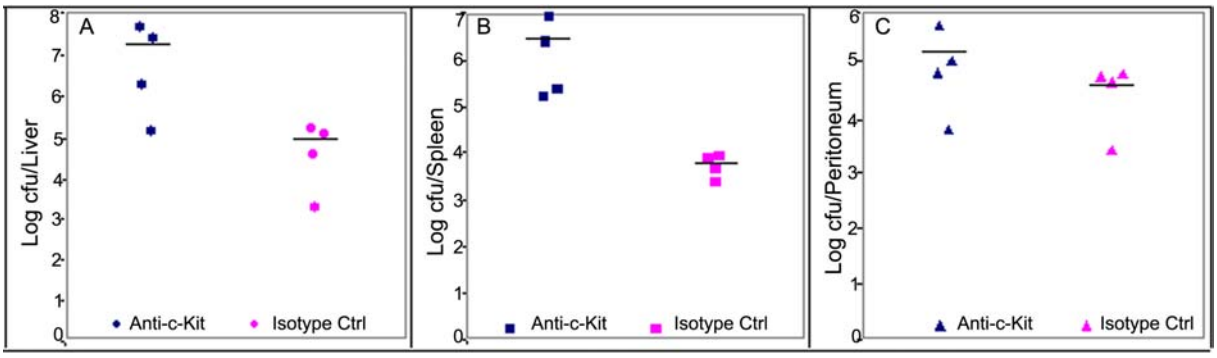

Figure 3.25. Mast cell depletion leads to an increase in susceptibility to *L. monocytogenes* infection. Normal (+Isotype control) and mast cell depleted (+anti-cKit) mice were infected with 1x10^5 WTL.m intraperitoneally and sacrificed at day 3 post infection to determine the bacterial burden in the liver (**A**), spleen (**B**) and peritoneum (**C**). The circles, squares, and triangles represent individual mice while the bars represent the geometric mean cfu/organ. Note the different scales in the panels.

In summary, the experiments described here show that in the sepsis model in which *L. monocytogenes* are injected in high doses intraperitoneally, mast cells have a significant role in the host defenses. Thus, mast cells, a cell type often associated with harmful hyper reactions such as allergy and autoimmune diseases, also have a definite beneficial function in the host in the combat against bacterial infections such as Listeriosis. Their strategic localization at the ports of entry of pathogens combined with the propensity to rapidly secrete a battery of proinflammatory mediators in response to several bacterial products, puts them at the frontline of the innate immune reactions critical for the effective control of bacterial infections such as *Listeria.*

3.4 Role of Toll-like receptors (TLR) in activation of mast cells by *L. monocytogenes*

As discussed earlier, LLO is the main virulence factor of *L. monocytogenes* which without, the bacteria can neither survive nor generate a protective immune response (1). This could be attributed to its role in promoting the bacteria's intracellular life cycle as well as the signals it induces in the host cells. The data presented above have defined calcium flux induction and lipid rafts aggregation as the mechanisms by which LLO triggers a variety host cell responses. In mast cells for instance, LLO triggers the release of a battery proinflammatory mediators such as TNF-α that induce the recruitment of neutrophils, the principal mediator *L. monoctogenes* clearance. As presented above however, LLO does not hold an absolute monopoly in signal induction by *L. monocytogenes*. The LLO deficient mutant (ΔhlyL.m) as well as the HKLΔhly were found to induce cytokine /chemokine gene expression and hence the recruitment of neutrophils to the site of infection. This suggests that in the absence of LLO, other *L. monocytogenes* products can compensate for some of the LLO induced proinflammatory signals.

As discussed earlier, Toll-like receptors (TLRs) transmit signals in response to microbial molecules that activate innate immune defences (199). TLR2, and TLR5 have been implicated in the recognition of *L. monocytogenes* (214;217;245). TLR2 is involved in the recognition of bacterial peptidoglycan, lipoteichoic acid and lipoproteins, while TLR5 recognizes bacterial flagellins. Since lipoteichoic acid can also be recognized by the TLR4, it is possible that TLR4 could also be involved. In any case, LLO like the other cholesterol dependent cytolysin (CDCs) have also been suggested to be agonist of TLR4 signalling (219;220) making it all the more likely that *L. monocytogenes* also triggers host responses via the TLR4.

3.4.1 Activation of NF-κB by *L. monocytogenes*

NF-κB is the key transcription factor via which TLR signalling activates the transcription of proinflammatory cytokines and chemokines. In addition to other transcription factors such as NFAT as shown above, LLO has also been shown to activate NF-κB (77;82). This illustrates the ability of *L. monocytogenes* to trigger disparate signalling pathways that converge at NF-κB activation via multiple cell

surface receptors. This could explain the redundancy in proinflammatory signals induced by LLO and ΔhlyL.m. To investigate such a possibility, first, whether LLO and ΔhlyL.m induce NF-κB activation in the BMMCs was investigated. To that end, BMMCs were stimulated with either WTL.m, ΔhlyL.m, or LLO for 45 min, fixed, then subjected to immunoflourescence staining to evaluate the intracellular localization of the NF-κB isoforms p50, p52 and p65. All of the above stimuli were found to induce nuclear transclocation of the already mentioned NF-κB isoforms. Figure 3.26 shows that NF-κBp50 and NF-κBp52 accumulate in the nuclei of cells stimulated with LLO and ΔhlyL.m but not the untreated control (bottom). A similar NF-κBp65 staining pattern was observed (data not shown).

3.4.2 Role of TLR signaling in the activation of proinflammatory cytokines /chemokine genes

Next, it was investigated whether the redundant proinflammatory signals induced by L. *monocytogenes* were due to NF-κB activation via the TLR signaling. For that, BMMCs from WT, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR2/4$^{-/-}$ mice were analyzed for their ability upregulate proinflammatory factors in response to WTL.m, ΔhlyL.m and LLO.

Again, analysis was focused on TNF-α, MIP-1α and MCP-1, three of the main factors that play an essential role in the innate defences against *Listeria.*

As shown earlier, RT-PCR analysis revealed that WTL.m, ΔhlyL.m and LLO do up regulate the mRNA of all of the above proinflammatory factors in the BMMCs from WT mice (Figure 3.27). Interestingly, both WTL.m and LLO but not ΔhlyL.m were found to induce these genes in the BMMCs from MyD88$^{-/-}$, TLR4$^{-/-}$, and TLR2/4$^{-/-}$ mice. (Figure 3.27). Although strong indications from analysis of TLR2$^{-/-}$ BMMCs suggest a similar gene activation pattern (data not shown), an accidental error is sample labelling necessitates a repeat of the experiment in order to ascertain those particular results. This was not possible within the time frame of this thesis. Notwithstanding the pending reanalysis of the TLR2$^{-/-}$ cells, the data presented herein do provide a basis for the following interpretation; *L. monocytogenes* activates TNF-α, MIP-1α. and MCP-1 genes via two independent signalling mechanisms; TLRs signalling and signal induction by LLO.

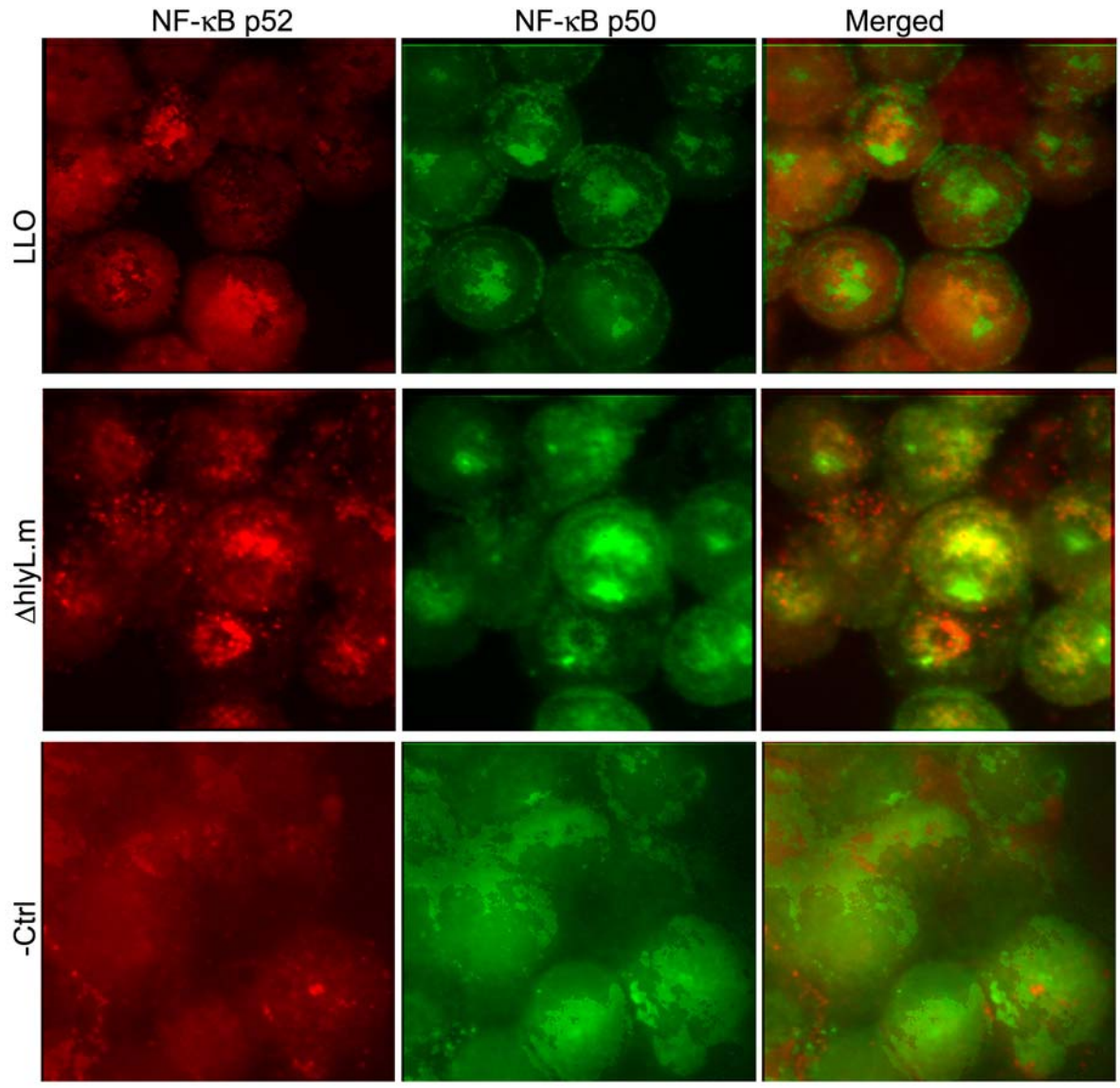

Figure 3.26. *L. monocytogenes* activates nuclear translocation of NF-κB via LLO dependent and independent mechanisms. BMMCs were incubated with (or without) either LLO (0.25 µg/ml) WTL.m or ΔhlyL.m (MOI 100) for 45 min, fixed, permeabilized and then stained with mouse anti-NF-κBp52 and rabbit anti-NF-κB p50. Subsequently, the cells were incubated with Cy3-goat anti mouse (red) and Alexa488-goat anti-rabbit (green). The panel in the left show the NF-κB p52 staining, the middle show NF-κBp50 while the right show a merger of the signals. Cells stimulated with WTL.m showed a similar pattern of NF-κB nuclear accumulation to that observed in LLO and ΔhlyL.m treated cells.

Thus in the absence of LLO, TLR signals do provide the proinflammatory signals induced by ΔhlyL.m in the WT cells whereas LLO provides the proinflammatory signals induced by WTL.m in TLRs deficient cells. This could explain why in the absence of both signals, ΔhlyL.m cannot induce gene activation in TLRs deficient mast cells.

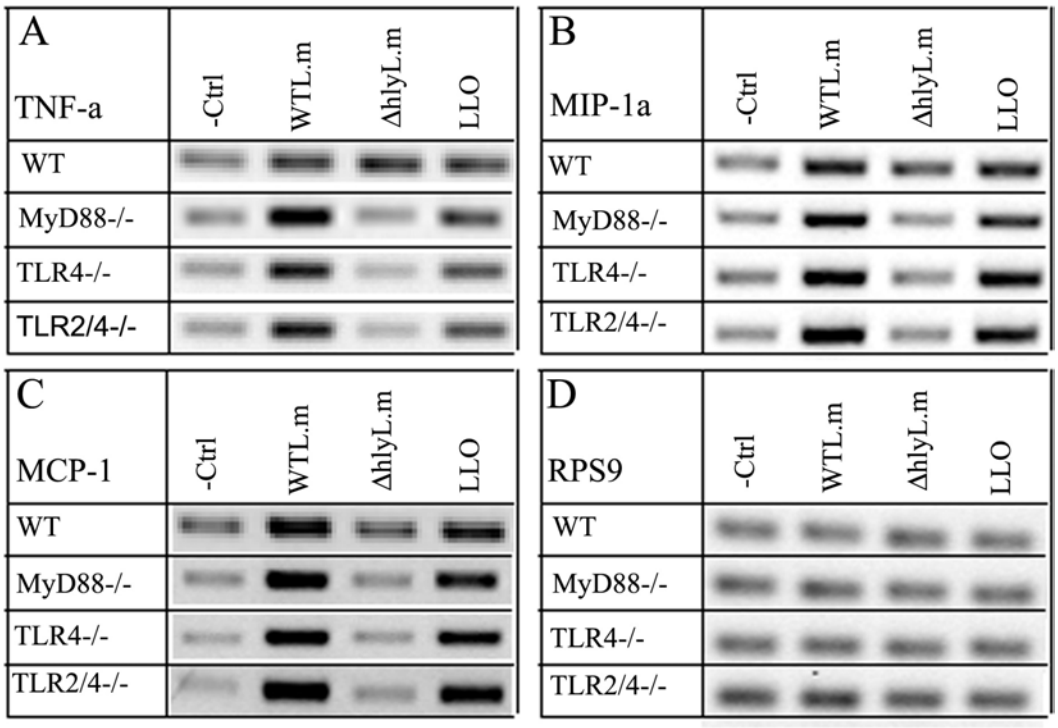

Figure 3.27. Role of LLO and TLR signaling in cytokine and chemokine induction by *L. monocytogenes.* BMMCs from WT, MyD88$^{-/-}$, TLR4$^{-/-}$ and TLR2/4$^{-/-}$ mice were incubated with either LLO (0.25ug/ml), WTL.m or ΔhlyL.m. After 3 h cells were analyzed RT-PCR for the mRNA of TNF-α, (**A**), MIP-1α (**B**), MCP-1 (**C**), and the house keping gene RSP9 (**D**).

Thus, taken together, these experiments impressively demonstrate how a complex pathogen like *L. monocytogenes* influences its survival in the host by employing different virulence factors and other bacterial components to trigger multiple but sometimes redundant signalling pathways.

4 Discussion

LLO is the main virulence factor of *L. monocytogenes* which without, the bacteria can neither survive nor generate a protective immune response. This could be attributed to its role in promoting the bacteria´s intracellular life cycle as well as the signals it induces in the host cells. The aims of the present study were to delineate the mechanisms of signal induction by LLO and to understand the significance of such signals *in vivo* during *Listeria* infection.

LLO can trigger signal in host cells via pore dependent and pore independent mechanisms. The present studies have defined calcium flux induction and lipid rafts aggregation as the pore dependent and pore independent mechanisms respectively, via which LLO triggers a variety host cell responses. To understand better the functional significance of signal induction by LLO, for most of the studies, focus was laid on mast cells, a cell type so far not investigated in the context of *Listeria* infection. In this cell type, LLO was found to triggers the release of battery proinflammatory mediators such as TNF-α that induce the recruitment of neutrophils, the principal mediator *L. monocytogenes* clearance. These findings have culminated in identifying mast cells as being a key player in the innate immune responses against Listeriosis. In addition to elucidating the mechanisms and role of LLO, effort was also extended towards understanding the role of TLRs in signal induction by *L. monocytogenes*. Initial data indicate that by triggering analogous signalling pathways, LLO and other listerial components that engage TLRs could compensate for each other in the induction innate proinflammatory reactions. The main highlights of the present findings are discussed in more details below.

4.1 The Influx and Efflux of Ca^{2+} induced by LLO in target cells- the consequence of double membrane perforation

In the present study, LLO, a member of the CDCs, was shown to cause the influx as well as the release of Ca^{2+} from the intracellular stores. As envisioned in the model presented in Figure 4.1, Ca^{2+} release from the intracellular stores might be due to the perforation of the ER membrane by LLO following the influx of LLO monomers via the

primary plasma membrane pores. The self-delivery of LLO into the cytosol has previously been shown (227). Although the possibility that LLO transported in endocytic vesicles via the retrograde transport could be delivered to the ER to cause Ca^{2+} release cannot also be excluded, the assumption of cytosolic LLO diffusing passively to the ER membrane appears to be more straightforward.

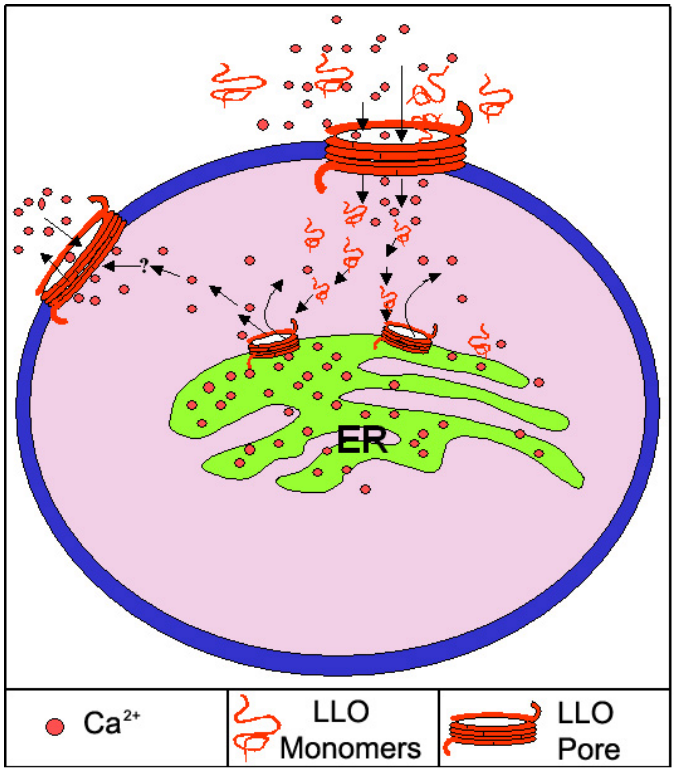

Figure 4.1. Schematic illustration of how LLO doubly penetrates the plasma and the intracellular membranes to cause the influx of Ca^{2+} as well as its release from intracellular stores. LLO monomers bind to the cell membrane and assemble into oligomeric pores large enough to allow the passage of smalls ions such as Ca^{2+} and proteins. The entry of LLO monomers into the cytosol via the primary transmembrane LLO pores subsequently lead to their assembly into pores on the cholesterol-rich internal membranes such as the ER. Whereas the release of intracellularly stored Ca^{2+} via these internal pores can trigger cellular responses such as cytokine induction, its sustained efflux due a prolonged incubation with LLO eventually leads to depletion of intracellularly stored Ca^{2+}.

The cytosolic delivery of LLO via a self-delivery mode is reminiscent of the 'type III' secretion system used by Gram-negative bacteria to deliver bacterial virulence factors into host cells. This view is in fact in concordance with the recent suggestion that cholesterol dependent cytolysins (CDCs) could be an analogous system in Gram-positive bacteria enabling them to deliver virulence factors into host cells (87). In this respect, the cytosolic delivery of PI-PLC, another virulence factor of *L.*

monocytogenes which also triggers Ca^{2+} release from intracellular stores, but via the generation of IP_3, is also mediated by LLO (88).

Induction of Ca^{2+} signals by *L. monocytogenes* in target cells is very important in the context of bacterial survival in the host. Ca^{2+} induction by LLO facilitates bacterial uptake in epithelial cells (71) while inhibiting uptake by macrophages (88). This illustrates how the pathogen strategically manipulates Ca^{2+} induction to maximize entry into the host via the epithelia, but minimizing its phagocytosis by bactericidal macrophages.

As demonstrated in this work for mast cells, Ca^{2+} induced by LLO can trigger the *de novo* synthesis and secretion of preformed proinflammatory mediators. This could facilitate bacterial spreading by recruiting more potential host cells to the site of infection. Similarly, other effects observed during *Listeria* infection like, induction of apoptosis in spleen or liver cells, might also involve Ca^{2+} fluxes induced by LLO (42;43).

While the current data do not rule out other mechanisms such as Ca^{2+} channels, the current data do at least suggest that release of Ca^{2+} from intracellular stores is most probably due to the passive efflux from the ER via the toxin pores. This uncontrolled release ultimately leads to the depletion of Ca^{2+} from such stores. Hence, cells pre-exposed to LLO are refractory to subsequent Ca^{2+} inductions. Depletion of intracellular Ca^{2+} stores in host cells may have important physiological significance in the context of listeriosis. The productive activation of lymphoytes requires a balanced integration of Ca^{2+} and other signalling pathways while stimulation of the antigen receptor in the absence of Ca^{2+} signalling or the vice versa, leads to anergy (246). Accordingly, when subjected to a sustained exposure to the Ca^{2+} ionophore ionomycin (an agent also known to deplete intracellular Ca^{2+} stores), T cells not only become refractory to subsequent Ca^{2+} induction via the antigen receptor, but generally exhibit an anergic state (247). In light of the present findings, it is imaginable that during *Listeria* infection, a prolonged exposure of host lymphocytes to LLO could render such cells unresponsive to stimulation, thus undermining the host's ability to mount an effective immune response, much to the pathogen's advantage.

That LLO doubly penetrates the plasma and the internal membranes provides a new perspective in our understanding of how the CDCs affect target cells. In fact, the implications thereof stretch far beyond the release of intracellular Ca^{2+} release as

shown in this study. Based on the current as well as other published findings (87;88), it would not be entirely groundless to speculate that LLO secreted by the intracellular bacteria probably also mediate the delivery of virulence factors into the cholesterol containing intracellular organelles, thereby modifying their functions.

Over time, cells can repair membrane lesions (248). Indeed, it was observed, that cells could recover from toxin attack and even restock the intracellular Ca^{2+} stores. Thus, LLO and the other CDCs provide attractive prospects in cell biology not only for introducing molecules into cells, but also for studying or manipulating Ca^{2+} regulated processes.

In conclusion, the data in this study provide a novel paradigm for the CDCs with important implications in the understanding of how these toxins contribute to the pathogenesis in their respective hosts.

4.2 LLO induces signalling in host cells via the aggregation of lipid rafts

In this part of the study, the co-aggregation of different rafts components was revealed as the second novel mechanism by which LLO triggers signalling in host cells. As illustrated in the proposed model below (Figure 4.2), rafts' clustering is due to oligomerization of membrane cholesterol bound toxin monomers. By using a cholesterol pre-inactivated form of LLO (CL-LLO) it was also demonstrate that LLO does activate tyrosine kinases in a pore independent manner.

Pore independent aggregation of rafts LLO is of important physiological significance in the context of listeriosis. *In vivo,* most of the LLO secreted into physiological fluids by extracellular bacteria or ruptured infected cells is most likely rapidly inactivated by blood borne cholesterol thus abrogating its ability to form pores as well as to trigger signals via such a mechanism. Therefore, the ability of CL-LLO to aggregate rafts guarantees that despite the loss of pore formation, the toxin can still trigger different cell-type specific responses, which ultimately influence the course of infection as discussed earlier.

Raft aggregation could also help to explain the differential signals induced by LLO in the various host cells. Since the signalling molecules associated with rafts differ from one cell type to the other, it is hereby hypothesized that the composition of rafts could dictate the cell type specific signals triggered by LLO. Thus as shown for Lyn in the

J774 macrophage line, LLO probably induces signalling in different cell types via the dominant rafts associated receptors, kinases and adaptors.

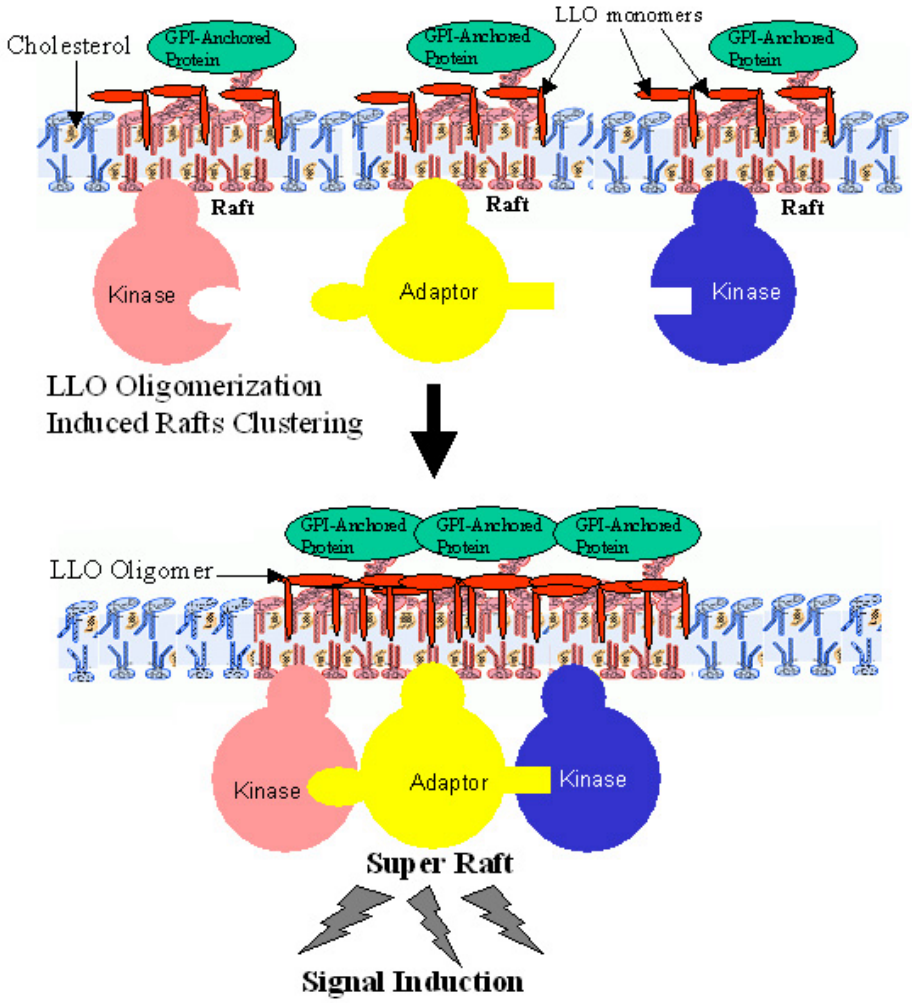

Figure 4.2. Model for rafts and signal transduction by LLO. LLO either binds directly to the cholesterol in rafts or is indirectly targeted to rafts by the cholesterol bound in solution. The oligomerization of rafts-associated toxin monomers (40–80), then results in the clustering of rafts leading to signal induction. The raft clusters formed thereof are large and can be visualized easily by light microscopy.

The results herein presented also have a number of implications for the role of cholesterol during the interaction of LLO (or CDCs in general) with the target membrane. The oligomerization and raft clustering data show that the cholesterol pre-inactivation step does not inhibit its subsequent oligomerization on target membranes. This finding was rather unexpected in face of electron microscopic observations from our laboratory documenting the absence of arc or ring shaped structures on erythrocyte ghosts treated with CL-LLO (66). The simplest explanation

that may reconcile these observations is that, whereas oligomerization and membrane insertion of LLO results in the formation of functional pores readily visible as arc or ring structures, oligomerization of CL-LLO on the other hand results in the formation of clandestine non-lytic oligomers probably similar to the streptolysin O (SLO) non-lytic oligomers (225) or the perfringolysin O (PFO) prepore complexes (249). One of the functions of cholesterol in the interaction of CDCs with target membranes is the induction of a conformational transition necessary for membrane insertion (250;251). It therefore seems feasible that in the aqueous environment, cholesterol triggers the toxin to adopt a faulty conformation, which although still capable of self-aggregation, cannot subsequently insert into the non-polar lipid bilayer.

In addition to demonstrating the role of LLO oligomerization on raft clustering and signalling, the data confirm a previous study that predicted the involvement of regions in the N terminal domain 1 in toxin oligomerization (234). However, the surprising finding that the treatment of the antibody neutralized LLO with cholesterol results in the restoration of oligomerization and hence rafts' clustering (Figure 3.13 and Figure 3.14), highlights an additional role of cholesterol in the complex process of oligomerization. First, it suggests that other self-aggregation sites do exist, and secondly, that self-aggregation via such sites is dependent on the conformational transitions triggered in the toxin monomers by cholesterol. Data based on proteolytic and recombinant fragments suggesting self-aggregation sites in the C-terminal half of the CDCs (252;253), are in keeping with the present finding, as are data showing that interaction with cholesterol triggers major conformational changes in the monomers that facilitates oligomerization and membrane insertion (250;251;254).

How does CL-LLO aggregates rafts' components? It is known from model membrane systems that due to Van der Waal forces, cholesterol and other saturated lipids tend to preferentially assemble into the hydrophobic lipid ordered microdomains as compared to the non raft matrix of the membrane (85). Thus, upon adsorption into the membrane, cholesterol exogenously introduced as a complex with LLO (i.e. CL-LLO) also partitions into rafts domains where the subsequent oligomerization of the bound LLO ultimately leads to the aggregation of not only cholesterol but other rafts components indirectly associated with it as well.

Co-clustering of raft components due to toxin oligomerization is probably not restricted to LLO or members of the CDCs as such. The recent findings that the

heptamerizing toxin aerolysin (183) does also co-cluster different GPI-anchored proteins (109) suggests that other oligomerizing toxins that engage receptors in lipid rafts probably also induce rafts` clustering. It however remains to be shown whether co-clustering by other toxins is only limited to their receptors (e.g. the GPI- anchored proteins in the case of aerolysin) or whether that also extends to the other indirectly associated rafts´ components as herein shown for LLO. In addition, the oligomeric complexes formed by CDCs are generally larger than those of any other toxins studied so far. Thus, the extent of rafts` clustering by such toxins probably pales in comparison to that by the CDCs.

In conclusion, the present study provide evidence that LLO is a potent inducer of raft aggregation, which might provide one of the molecular explanations for the broad host cell responses, mediated by LLO during *Listeria* infection. The findings could also provide a general paradigm of how other CDCs might interact with target cells.

Equally important, the findings herein demonstrate the potential application of LLO in studying the biology and composition of rafts. Although lipid rafts have been implicated in a variety of biological functions (94;95), their structure, with respect to size and composition is still uncertain. Recent studies based on single particle tracking (255) and fluorescence resonance energy transfer (FRET) (109;110;256) methods have placed the size of rafts at a nanometer range explaining why native rafts have eluded detection by standard light microscopy. Thus microscopical visualization of rafts has only been accomplished after aggregating rafts` components like by the use of antibodies (110;111). However, owing to the tendency of raft components to segregate into distinct raft sub-types, attempts to use antibodies for the co-clustering of different rafts components have largely been unsuccessful (109;110;257). Thus, since LLO indiscriminately co-clusters of rafts` components by aggregating membrane cholesterol (the universal “glue” of rafts), it could therefore be used to visualize rafts and hence identify putative rafts component as well as signalling pathways mediated via rafts.

4.3 Role of mast cells in the control of *L. monocytogenes* infection

The functional significance of signal induction by LLO was investigated in mast cells. LLO was found to induce two important responses in this cell type (1) rapid secretion of pre-sythesized stored inflammatory mediators via degranulation and (2) *de novo* synthesis of such mediators. Using a *Listeria* septic peritonitis model, the data herein presented show that the secreted mast cell products especially those with neutrophil chemo-attractive properties play a critical role in host defence against *L. monocytogenes.* The specific contribution of mast cells to the anti-*Listeria* innate immune defence was demonstrated by the depletion of mast cells using a monoclonal antibody against c-Kit, a protein highly expressed on mast cells. Mast cell depleted mice showed a significantly diminished capacity to attract neutrophils following intraperitoneal challenge with *L. monocytogenes*. As previously shown for other bacterial pathogens (32), these recruited neutrophils were found to be critical for early clearance of bacteria at the site on infection thus limiting dissemination to other organs such as the liver and spleen. In these organs, the number of *Listeria* in the mast cell depleted mice was found to be more that 2 logs higher as compared to the undepleted controls. These data are highly consistent with findings from other models of infectious peritonis using the mast cell deficient W/W^v mice (28;32)

Efforts to understand the mast cell secretory products that orchestrate the rapid recruitment of neutrophils into the peritoneum led to the identification of TNF-α. Of the multiple chemo-attractants that can be released by mast cells, TNF-α was considered of special interest because it is one of the presynthesized mediators stored in the mast cell granules hence is rapidly released upon activation (30;237;238;258). Accordingly, inoculation of *L.monocytogenes* into the peritoneum was found to induce a rapid accumulation of TNF-α in the peritoneal cavity in a mast cell dependent manner. Administration of TNF-α alone into the peritoneum induced a rapid influx of neutrophils thus confirming the direct role of TNF-α in such a response. That being the case however, it must be emphasized that these findings do not rule out the involvement of other mast cell derived neutrophil chemoattractants. Indeed TNF-α, may act in an auto/paracrine fashion and stimulate release of other

chemokines such as macrophage inflammatory protein 2 (MIP-2), or IL8 – also potent neutrophils chemoattractants (259). Additionally, TNF-α can facilitate neutrophil extravasation through endothelial walls by triggering endothelial cell expression of various cell adhesion molecules such as endothelial-leukocyte adhesion molecule (ELAM)-1 (30;31;240;260). TNF-α is also known to enhance the bactericidal activities of neutrophils (239).

Besides controlling bacterial infection via neutrophils, mast cells can also control bacteria via direct mechanisms. Mast cells are also known to bind to and phagocytose bacteria (261) and release anti microbial peptides (262;263). Electron microscopic evaluation showed that compared to macrophages, internalisation of *Listeria* by BMMCs is extremely poor. This suggests that phagocytosis plays a minor role in listerial clearance.

Although the present findings unequivocally show that mast cells play a critical role in host defence against *L. monocytogenes* infection, they are slightly at odd with one of the recent findings. The recent study by Edelson et al (264) using the mast cell deficient W/W^v mice found identical *Listeria* burdens in both spleens and livers of W/W^v and mast cell reconstituted W/W^v mice. Interestingly, and consistent with the present findings however, mast cells were demonstrated to play an important role in the recruitment of neutrophils into the peritoneum in response to *Listeria*. A number of reasons could be responsible for these disparate findings. First, the difference in the infection dose and the genetic background of the mice used. In the present study an intraperitoneal dose of $1x10^5$ *Listeria*/mouse compared to $5x10^4$ in that particular study. Although these doses may not be very different *per se*, it has to be borne in mind that compared to the BALB/c mice employed in the present study the C57BL/6 X 129/Sv background of W/W^v mice is highly resistant to *Listeria* infection. Secondly, the intrinsic difference in the two systems i.e reconstitution of mast cell deficient mice verses depletion of mast cells could influence the ultimate readout of the mast cell function under evaluation. For instance, as opposed to the high efficiency of mast cell depletion (over 90 %) in the present study, the fact that reconstituted mice contained only 10 to 20% of the normal peritoneal mast cells numbers could in part account for the modest mast cell-dependent anti-*Listeria* host defence observed in that particular study.

In summary, the data presented herein show for the first time that mast cells, a cell type often renowned for its distressing contribution to chronic inflammatory diseases

(e.g, asthma) also has a primary role in the innate immune defences against *L. monocytogenes.*

4.4 Role of TLRs signalling in the activation of mast cells by *L. monocytogenes*

The overall activation of host cells by complex pathogens such as *L. monocytogenes* clearly involves the coordinated and often redundant activation of signalling pathways by several bacterial components. Thus it is difficult to ascertain the specific contribution of a particular product by analysing the host response to the whole organism. This is best illustrated by the fact that cytokine and chemokine expression and the consequential recruitment of inflammatory cells could be induced independently by LLO and ΔhlyL.m. As discussed above *L. monocytogenes* can induce signalling in host cells via TLRs such as TLR2, TLR4, TLR5, TLR6 and TLR9 (213;216-218;245). Evaluation of mast cells from MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$ and double TLR2/4$^{-/-}$ showed that while these receptors seem to play a role in cytokine and chemokine transcriptional activation by ΔhlyLm, apparently they are dispensable in the induction of such a response by WTL.m and LLO. This means that at least in mast cells, LLO and other listerial components that engage the TLRs, trigger redundant proinflammatory signals. Based on the present data by which LLO and ΔhlyL.m were shown to induce the activation of NF-κB, a master regulator of several proinflammatory genes, it is tempting to hypothesize that the redundancy in the proinflammatory responses induced by LLO and ΔhlyL.m is in part due to the convergence of signalling pathways activated by these stimuli. In addition to NF-κB, activation of the transcription factor AP-1 via the MAPK signalling pathways is also a potential common point of convergence between signal induction by LLO and ΔhlyL.m. This is supported by the several finding showing LLO and signal induction via TLRs can also activate this pathways (68;69;197;265).

Redundancy in the induction of proinflammatory responses in host cells by *L. monocytogenes,* and the possibility thereof that the proinflammatory signals triggered via the TLRs could be compensated for by other listerial products such as LLO, may help to explain the controversial findings with respect to the role of TLR2 in the host defence against Gram-positive bateria that secrete CDCs (213;214;245;266-268). Although there abundant evidence from *in vitro* observations that TLR2 is the

predominant receptor signalling the presence of cell wall components of Gram-positive bacteria, such as peptidoglycan or lipoteichoic acid, data that confirm these observations *in vivo* are still scarce. In the case of *L. monocytogenes,* studies carried so far suggest that it is either dispensable or that its contribution to the control of *Listeria* is only modest (213;214;245). Similarly, a recent study has shown that TLR2 does not importantly contribute to host defence in the *Strepococcus pneumoniae*. To add on to the complexity of the role of TLRs in the innate immune responses to Gram- positive bacteria, recent studies have reported that the CDCs signal via TLR4 (219;220). Hence TLR4 mutant mice demonstrated an increased mortality when infected with wild type but not pneumolysin deficient pneumococci.

Efforts are currently underway to investigate the signalling pathways common to LLO and TLRs and how this redundancy in the activation of proinflammatory signals by LLO and TLR ligands contributes to the overall host defences against *Listeria.*

Taken together the present study add several novel findings on how *L. monocytogenes* interacts with its host which should spark off a plethora of further experiments that should finally lead to systems biology of listeriosis.

5 Summary

The Gram-positive bacteria *Listeria monocytogenes* is the food borne etiological agent of Listeriosis whose manifestations include septicemia, meningitis, encephalitis and abortions in humans and livestock. The ability of *L. monocytogenes* to infect and survive in a wide range of animal species is attributed to several virulence factors which enables it to infect and proliferate in a variety of host cells. The pore-forming toxin, Listeriolysin O (LLO), is considered to be the most important virulence factor of *L. monocytogenes*. In addition to playing the essential role of allowing the bacteria to cross membrane barriers during its intracellular life cycle, LLO also act as a pseudo cytokine / chemokine, which induces a broad spectrum of host responses that ultimately influences the outcome of listeriosis. How LLO triggers signalling in host cells has been a subject of intense debate. It is now clear that LLO can trigger signalling in the host cell via pore dependent and pore independent mechanisms.

The present data show that the pore dependent mechanism of signal induction by LLO is in part due its ability to cause influx of extracellular Ca^{2+} as well as release from intracellular stores in host cells. The data presented suggest that LLO accomplishes this by first forming transmembrane LLO pores which then allow influx of Ca^{2+} as well as LLO molecules into the cytosol. The cytosolic LLO molecules then form secondary pores in membranes of internal organelles such as the endoplasmic reticulum to cause the release of the intracellularly stored Ca^{2+}.

Secondly, the present study shows that the pore independent mechanism of signal induction by LLO is due to the aggregation the lipid rafts and their associated signalling molecules. Since cholesterol is the main structural component of lipid rafts, the cholesterol dependent cytolysin LLO binds to cholesterol in such membrane microdomains and spontaneously aggregates them as a result of oligomerization.

The functional significance of signalling by LLO was evaluated in mast cells. The *in vitro* and *in vivo* data presented show that LLO induces the spontaneous release of presynthesized proinflammatory factors as well as their *de novo* synthesis. It is herein shown that the release of such proinflammatory mediators following activation of mast cells by LLO or *L. monocytogenes* triggers robust innate host reactions necessary for the control of *Listeria* infection. As a result of these finding it herein shown for the first time that mast cells, a cell type renowned for allergic and

autoimmune reactions also plays an important role in the control *L. monocytogenes* infection.

To gain a more wholesome understanding of the mechanisms of signal induction by the whole organism, in the present work, the role of Toll-like receptors (TLRs) in the proinflammatory signals by *L. monogytogenes* was also evaluated. The findings indicate a redundancy in the proinflammatory signals induced by LLO and other listerial components that signal via the TLRs. Thus, although listerial cell wall components do trigger signalling via TLRs, such signals seem to be dispensable as they can be compensate for by LLO. These findings could help to reconcile the several controversial findings with respect to the role of TLRs in the innate immune reactions against Gram-postive bacteria that express cholesterol dependent cytolysins such as LLO.

In overall, the findings impressively demonstrate how a complex pathogen like *L. monocytogenes* influences its survival in the host by employing different factors to trigger multiple but sometimes redundant signaling pathways.

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